SALMONELLA TYPHIMURIUM GROEL EXPRESSED ON THE CAPSID OF THE T7 BACTERIOPHAGE: A STUDY OF INDUCED HUMORAL IMMUNITY IN MICE

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TABLE OF CONTENTS

FIGURES AND TABLES

Tables:

LIST OF ABBREVIATIONS

Amp^R	ampicillin resistant
ADP	adenosine diphosphate
AEBSF	4-(2-aminoethyl) benzenesulphonylfluoride
Ahp	alkyl hydroperoxidase
APC	antigen presenting cell
APS	ammonium persulphate
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
bp	basepairs
BME	bacterial-mediated endocytosis
BPI	bactericidal permeability increasing protein
BSA	bovine serum albumin
°C	degrees Celsius
CD	cluster of differentiation
CDR	complementarity determining region
c.f.u.	colony forming units
Ci	Curies
CMI	cell-mediated immunity
Cml^R	chloramphenicol resistant
CTL	cytolitic T lymphocyte
dCTP	deoxycytidine triphosphate
Da	Daltons
DAP	diaminopimelic acid
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Dps	DNA binding protein
DTH	delayed-type hypersensitivity
DT104	definitive phage 104
DTT	dithiothreitol

EDTA	ethylenediaminetetraacetate
ELISA	enzyme linked immunosorbent assay
F_C	carboxyl terminal region of immunoglobulin
g	grams
g	standard acceleration of gravity
dGTP	deoxyguanosine triphosphate
GALT	gut-associated lymphoid tissue
Gly	glycine
hil	hyperinvasion locus
HRP	horseradish peroxidase
HSP	heat-shock protein
dH_2O	distilled water
IFN	interferon
Ig	immunoglobulin
sIgA	secretory immunoglobulin A
IL	interleukin
i.p.	intraperitoneally
IPTG	isopropyl-β-D-thiogalactoside
i.v.	intravenously
IVET	*in vivo* expression technology
kb	kilobases
l	litres
LB	Luria-Bertani medium
LD_{50}	50% lethal dose
LPS	lipopolysaccharide
LSB	Laemmli (loading) sample buffer
M	molar
MBP	major basic protein
MDR	multidrug resistant
Met	methionine
mA	milliamps
mg	milligrams

MHC	major histocompatibility complex
ml	mililitres
mm	millimetres
MW	molecular weight
µl	microlitres
N	normal
NADH	nicotinamide adenine dinucleotide (reduced form)
NO	nitric oxide
Nramp	natural resistance associated macrophage protein
dNTP	deoxynucleotide triphosphate
NK	natural killer
OD	optical density
OMP	outer membrane protein
OPD	*O*-phenylenediamine
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
p.f.u.	plaque-forming units
PMN	polymorphonuclear leucocyte
PMSF	phenylmethylsulphonyl fluoride
PPD:	purified protein derivative
prg	PhoP repressed genes
RES	reticuloendothelial system
RNA	ribonucleic acid
RNase	ribonuclease
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid
ROS	reactive oxygen species
rpm	rotations per minute
SDS	sodium dodecyl sulphate

SEF	*S. enteritidis* fimbriae
sif	*Salmonella*-induced filaments
sip	*Salmonella* invasion proteins
SLE	systemic lupus erythematosus
Sod	superoxide dismutase
sop	*Salmonella* outer proteins
SP	spacious phagosome
spa	surface presentation antigen
SPI	*Salmonella* pathogenicity islands
STF	*S. typhimurium* flagella
STM	signature-tagged mutagenesis
TB	terrific broth
TBS	tris buffered saline
TCR	T cell receptor
TEMED	N, N, N'-tetramethylethylenediamine
Tet^R	tetracycline resistant
T_H	helper T cell
TNF	tumour necrosis factor
T-TBS	Tween - tris buffered saline
dTTP	deoxythymidine triphosphate
Tris	tris(hydroxymethyl)aminomethane
Tween 20	polyoxyethylene sorbitan monolaurate
UDP	uridine 5'-diphosphate
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-indoyl-β-D-galactoside

ABSTRACT

The massive occurrence of diarrhoeal disease due to *S. enterica* infection, the increased incidence of typhoidal mortality, and also the recent emergence of *Salmonella* multidrug-resistant (MDR) strains, underline the important role for the design of preventive immunisation strategies. Because early vaccines elaborated against *S. enterica* showed a low efficacy, the need for improved vaccines has emerged, which would stimulate the participation of both cellular and humoral immunity. GroEL was shown to be an immunodominant antigen and a major immunogen of many bacterial pathogens. When *S. typhimurium* is subjected to environmental stress, such as the intracellular milieu of the macrophage, the bacterium responds by inducing 30-40 proteins, and GroEL is among them. Elimination of *S. typhimurium* from its host involves the generation of a T_H1 response, and recent work performed in our laboratory (Taylor, 1997) has shown that *S. typhimurium* GroEL is able to induce significant humoral and DTH responses, suggesting that this protein is recognised in the context of a T_H1 immune response.

Bacteriophages offer the advantage of non-replication in the host over animal viruses for foreign protein display and when genetically modified, are highly immunogenic and induce rapidly specific anti-bacteriophage antibodies. The hybrid T7 bacteriophage was used in this study for the display of *S. typhimurium* GroEL fused in-frame to the 10B minor capsid protein of the phage, as a vehicle to deliver GroEL to the immune system of BALB/c mice. The ability of this construct was investigated for the induction of anti-GroEL humoral immunity, after i.v. injection in a two-dose regimen, 14 days apart. Due probably to the low copy number of 10B-GroEL estimated at less than 10% of the total capsid protein, this immunising agent barely stimulated detectable levels of anti-GroEL antibodies. These responses were significantly weaker than the anti-GroEL antibody levels induced in mice injected i.p. either with the purified *S. typhimurium* GroEL in the presence of alum, or with the *aroA*$^-$ attenuated *S. typhimurium* strain (MPG479). This hybrid T7 bacteriophage

to function as an immunising agent, probably because of insufficient antigen presentation and of administration in the absence of an adjuvant. However, detectable secondary antibodies (measured six and twelve days after challenge) directed against the major 10A capsid protein of the phage were present after immunisation with the hybrid phage, suggesting that the T7 phage is immunogenic by itself.

Prior to immunising BALB/c mice, the hybrid T7 bacteriophage was purified through a CsCl step gradient. This study reports the contamination of the hybrid T7 particle with the exogenous, cellular GroEL protein from the *E. coli* host of the phage, regardless of the heterologous protein expressed on the phage capsid. However, it provides a solution for the de-contamination of the T7 CsCl-purified particle, by precipitation of the phage with 1% SDS, an anionic detergent which has the capacity of disrupting protein complexes, and leaves the phage viable. The present work reports also the full DNA sequence of the *groEL* gene from *S. typhimurium* SL1344 chromosome.

CHAPTER 1

Introduction

1.1 *SALMONELLA* AND SALMONELLOSES

1.1.1 Bacteriology of *Salmonella*

Salmonellae are named after the pathologist Salmon who first isolated *S. choleraesuis* from the porcine intestine. They are widely dispersed in nature, including the gastrointestinal tracts of domesticated and wild mammals, reptiles, birds and insects. They are effective commensals, as well as pathogens that cause a spectrum of diseases (Miller and Pegues, 2000). The genus *Salmonella* is a member of the family *Enterobacteriaceae*. Its closest known relative is *Citrobacter* and other close relatives include *Escherichia*, *Shigella*, *Klebsiella* and *Enterobacter* (Le Minor, 1984; Farmer, 1995). *Salmonella* are Gram-negative, non-spore-forming, motile, facultatively anaerobic bacilli (Le Minor, 1984). Most salmonellae are aerogenic, producing gas from glucose, although *S. typhi* is an important exception. Most salmonellae produce hydrogen sulfide, but a few types, such as most strains of *S. paratyphi A*, do not (Le Minor, 1984).

Based on the principle that bacteria, which are related by more then 70% on the basis of DNA/DNA hybridisation studies, belong to the same "genospecies", all *Salmonella* isolates are classified as a single species (Crosa *et* al., 1973) and given the name *S. enterica.* This species can be sub-classified into eight subspecific groups, based on DNA similarity and host range (Groisman and Ochman, 2000). Members of each sub-group can be serotyped into more than 2,300 serovars according to somatic O antigens, surface capsular polysaccharide (Vi), and flagellar (H) antigens, which determine the organisms' reaction to specific antisera (Popoff *et* al., 1996; Groisman and Ochman, 2000; Miller and Pegues, 2000). For example, *S. typhi* and *S. paratyphi C* produce the polysaccharide Vi antigen, which is a homopolymer of N-acetyl galactosaminouronic acid (Daniels *et* al., 1989). Most antigenic variability occurs in the O antigen, which is composed of chains of oligosaccharide repeats, attached to a core oligosaccharide, which in turn is linked covalently to lipid A (Gray, 1995).

For the purpose of this thesis, the use of the genus name, *S. enterica*, will be used when referring to or discussing general properties or features of the serovars that form the genus. Serovars of *S. enterica* will de identified initially by their official designation, but subsequently referred to by their commonly used serovar name, *e.g. typhimurium*, which will be displayed in adjacent brackets.

Some *Salmonella* serovars are strictly adapted to one particular host whilst others are ubiquitous (Le Minor, 1984). Some serovars are highly adapted to humans and have no other known natural hosts (Rubin and Weinstein, 1977), such as serovar Typhi (*S. typhi*), serovar Paratyphi (*S. paratyphi*) and *S. sendai*, and usually cause grave diseases with septicaemia-typhoidic syndrome. In general, they are not pathogenic for other animal species. Other serovars are adapted to one animal species. For example, *S. abortusovis* is a major cause of abortion in ewes, and *S. typhisuis* and *S. gallinarum* (*S. pullorum*) are adapted to swine and poultry, respectively. Some salmonellae such as *S. dublin* (cattle) and *S. arizonae* (reptiles), are associated mainly with a particular species, but occasionally infect humans (Waterman *et* al., 1990; Fang and Fierer, 1991). Other organisms such as the ubiquitous *S. enterica* serovar Typhimurium (*S. typhimurium*) have a broad host range and can infect a wide variety of animal hosts. As such, these are a frequent cause of food-borne infections in humans and cause a typhoid-like disease in mice. The widespread distribution of salmonellae in the environment, their increasing prevalence in the global food chain, and their virulence and adaptability result in an enormous medical, public health and economic impact world-wide.

Diseases resulting from *S. enterica* infection

The diseases associated with infection by *S. enterica* are collectively termed salmonelloses. Specific *Salmonella* serovars most often produce characteristic clinical manifestations of gastroenteritis (food-poisoning), enteric fever, bacteremia and vascular infection, localised infection or development of chronic carrier state (Miller and Pegues, 2000). One of the most important health problems faced nowadays by the whole world comes from the high incidence of diarrhoeal diseases

(Maurice, 1994; Pang *et* al., 1995). Recent statistics issued by the World Health Organisation (WHO), have reported that approximately 3 million deaths result annually from the estimated 1.3 billion incidences of acute gastroenteritis/diarrhoea due to non-typhoidal salmonellosis (Pang *et* al., 1995).

Non-typhoidal salmonellae also cause a small but significant proportion of diarrhoea in travellers and in young children in developing countries (Molbak *et* al., 1994) and the most common serotypes isolated from human sources were *S. enteritidis* and *S. typhimurium* (Miller and Pegues, 2000). In humans, non-typhoidal *Salmonella* infections are often associated with food products and such infections are the second most frequently identified cause of food-borne disease outbreaks (Todd, 1997). Foods of animal origin, including meat, poultry, eggs or dairy products, are recognised vectors for *Salmonella* infections. Many countries have also become infected with *S. hadar* introduced by imported turkeys, or with *S. agona* introduced by fish meals imported from South America (Le Minor, 1984). *S. enterica* infection of animals such as chickens, calves, swine and sheep, has economic consequences in farming and animal husbandry (Coynault *et* al., 1996).

The ingested dose is an important determinant of the incubation period, symptoms and disease severity of salmonellosis. Data from outbreaks of salmonellosis suggest that, under some circumstances, even a low inoculum (less than 10^3 organisms) may produce non-typhoidal gastroenteritis (Blaser and Newman, 1982). Infection with non-typhoidal *Salmonella* most often results in self-limited acute gastroenteritis manifested by nausea, vomiting and diarrhoea within 6 to 48 hours after the ingestion of contaminated food or water. Fever (38^0 to 39^0C), abdominal cramping and chills are frequently reported. Headache, myalgia and other systemic symptoms may also occur. The diarrhoea is usually self-limited, typically lasting for 3 to 7 days and after resolution of gastroenteritis, the mean duration of carriage of non-typhoidal salmonellae in the stool is 4 to 5 weeks, but varies with serotype (Miller and Pegues, 2000).

Enteric fever

Human typhoid and paratyphoid fevers are severe systemic illnesses characterised by clinical syndromes of abdominal pain and fever distinct from acute gastroenteritis, and are collectively termed enteric fever. It has been estimated that each year more than 33 million cases (nearly 600,000 deaths) occur world-wide due to typhoid fever (Ivanoff and Levine, 1997). The disease is endemic in many developing countries, particularly the Indian subcontinent, South and Central America and Africa, with annual incidence rates estimated to be as high as 900 per 100,000 population in Asia (Ivanoff, 1994). In endemic areas the incidence of *S. typhi* infection is highest in children older than 1 year and probably reflects their lack of acquired immunity (Thikyakorn *et* al., 1987). Recently, mortality rates of 10 to 30% have been reported in certain Asian and African countries and have been associated with multi-drug resistant strains and delays in antibiotic therapy (Arand *et* al., 1990; Bhutta *et* al., 1991; Carmeli *et* al., 1993; Sugandhi *et* al., 1993).

Enteric fever is caused by *S. typhi*, but a less severe syndrome, paratyphoid fever, is caused by *S. paratyphi A*, *B* and *C*. Acquisition of organisms occurs by ingestion of food or water contaminated with human excreta. Usually, water borne transmission involves the ingestion of fewer microorganisms and, as a result, has a longer incubation period and lower attack rate than food borne transmission. With the introduction of water treatment in the 20th century, including sand filtration and chlorination, the incidence of typhoid fever waned massively in the developed world, but remained endemic in less developed areas, where faecal contamination of water sources still occurs (Plotkin and Orenstein, 1999; Miller and Pegues, 2000). Since the beginning of 1990s, 12% of multi-drug resistant strains of *S. typhi* with plasmid-encoded resistance to chloramphenicol, ampicillin and trimethoprim have emerged in the Indian subcontinent, Southeast Asia and Africa (Threlfall *et* al., 1997), compared to 0.6% during 1984 to 1989 (Miller and Pegues, 2000).

Treatment and prevention

Most symptoms of typhoid fever resolve by the fourth week of infection without antimicrobial therapy in the approximately 90% of patients who survive (Miller and

Pegues, 2000). Where antibiotic treatment is recommended, chloramphenicol has been the treatment of choice for typhoid fever since its introduction in 1948, but since 1970s, this therapy has been associated with the emergence of plasmid-mediated resistance (Paniker and Vimala, 1972; Olarte and Galindo, 1973; Lampe *et* al., 1974; Bhutta *et* al., 1991). This prompted the use of amoxicillin and trimethoprim-sulfamethoxazole, but recent emergence of multidrug-resistant (MDR) strains of *S. typhi* diminished the efficacy of these drugs (Threlfall *et* al., 1997). In areas with high prevalence of MDR *Salmonella* infection, patients suspected of having typhoid fever should be treated with quinolones or third-generation cephalosporins (ceftriaxone). Also for patients who are chronic carriers of *S. typhi*, the treatment of choice is ceftriaxone or ciprofloxacin, but also cholecystectomy may be necessary. Chromosomal- and plasmid-encoded resistance is emerging not only among human, but also among animal *Salmonella* strains (Anand *et* al., 1990; Heisig, 1993; Griggs *et* al., 1996).

Of particular interest is the recent emergence of a distinct strain of multidrug-resistant *S. typhimurium* in the U.K., characterised as definitive phage 104 (DT104), that is resistant to five antibiotics such as ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines. *S. typhimurium* DT104 is the second most prevalent *Salmonella* strain in the U.K. and resistance to trimethoprim and fluoroquinolones are also emerging (Threlfall *et* al., 1997). Infection with *S. typhimurium* DT104 has been associated with contact with ill farm animals and exposure to meat products (Wall *et* al., 1995). The present and anticipated future problems with multidrug-resistant salmonellae underline the vital role of preventive immunisation and make a strong motivation for the present study.

1.2 INTERACTIONS OF *S. ENTERICA* WITH HOST CELLS

1.2.1 Pathogenesis of enteric fever

S. typhi and *S. paratyphi* are highly invasive bacteria that pass through the intestinal mucosa of humans rapidly and efficiently to eventually reach the network of tissue macrophages, endothelial cells and polymorphs, known as the reticuloendothelial system (Levine *et* al., 1983; Roitt *et* al., 1998).

Ingested salmonellae firstly pass through the acid barrier of the stomach, which is the first line of defence against enteric infections (Giannella *et* al., 1972; Blaser and Newman, 1982). Organisms survive poorly at normal gastric pH (less than 1.5), but they can develop an adaptive acid-tolerance response that may promote survival at low pH (Foster and Hall, 1990; Gorden and Small, 1993). After leaving the stomach, the bacilli reach the small intestine and rapidly penetrate the mucosal epithelium by one of two alternative mechanisms to arrive in the *lamina propria* (Brandtzaeg, 1989). Secretory products of the intestine (*e.g.*, antimicrobial defensin-like peptides secreted by Paneth cells), pancreas and gallbladder (*e.g.*, bile salts) (Lehrer *et* al., 1991; Selsted *et* al., 1992), secretory IgA and intestinal mucus may play roles in preventing penetration of the enterocytes that line the intestine (Michetti *et* al., 1992). One mechanism of invasion involves active uptake of typhoid bacilli by the microfold cells (M cells), and other organised lymphoid tissue of the gut (Plotkin and Orenstein, 1999). From here they enter the underlying lymphoid cells. The second invasive mechanism consists in internalisation of typhoid bacilli by enterocytes through membrane-bound vacuoles that pass through the cell and ultimately release the bacteria at the basal portion of the cell without destroying the enterocyte (Takeuchi, 1967; Plotkin and Orenstein, 1999). After reaching the *lamina propria* in the non-immune host, typhoid bacilli rapidly interact with macrophages and lymphocytes in Peyer's patches and other lymphoid tissues located in the small intestinal sub-mucosa (Hackett *et* al., 1986). Recruitment of additional mononuclear cells and lymphocytes can result in marked enlargement and necrosis of the Peyer's

patches after several weeks of infection, which may be the cause of the characteristic abdominal pain (Rubin and Weinstein, 1977; McCormick *et* al., 1995). The ability of salmonellae to survive within macrophages is likely to be essential to typhoid fever pathogenesis and spread of the organisms to the systemic circulation.

Clinical illness occurs only when a critical number of organisms have replicated and is accompanied by a fairly sustained secondary bacteremia. Eventually, organisms are taken up by tissue macrophages in the bone marrow and Peyer's patches, but particularly in the liver and spleen (Hornick *et* al., 1970). Symptoms such as fever and abdominal tenderness may result from the secretion of cytokines by macrophages in response to bacterial infection (Edelman and Levine, 1986).

1.3 EXPERIMENTAL METHODS FOR INVESTIGATING *S. ENTERICA* INTERACTIONS WITH HOST CELLS

Many of the features and the outcome of *S. enterica* infection are dependent upon interaction with epithelial and phagocytic cells, especially macrophages. Pathogenic salmonellae can be studied in convenient *in vivo* and *in vitro* models that offer a good environment for understanding the events that occur during host-infection by *S. enterica*. Such models are being used to identify *Salmonella* genes required for virulence (Chatfield *et* al., 1992).

The mouse model of *S. enterica* infection

Many mice strains are highly susceptible to infections by isolates of several serotypes including *S. typhimurium*, *S. dublin* and *S. enteritidis* (Collins, 1974). These mouse virulent isolates normally cause invasive, systemic infections, which in many ways resemble typhoid. The murine model is often referred to as mouse typhoid or murine salmonellosis. However, mice do not develop gastroenteritis or diarrhoeal disease and cannot be used as a model for food poisoning (Chatfield *et* al., 1992).

Perhaps one of the most useful properties of the mouse model is the amenability to examination of *Salmonella* genes involved in the overall virulence process. Strains of *S. typhimurium* carrying mutations in the genes of interest with possible involvement in virulence can be compared against an otherwise isogenic parental strain, using the LD_{50} test (50% lethal dose). Although the murine model is readily accessible and is yielding valuable information, caution must be taken when extrapolating data obtained in the mouse to other hosts (Chatfield *et* al., 1992).

A genetic locus in mice that correlates with susceptibility to *Salmonella* infection

It has been known for some time that host resistance to murine salmonellosis is under genetic control (Robson and Vas, 1972; Plant and Glynn, 1976). With inbred mice, susceptibility to *S. typhimurium*, *Mycobacterium sp.* and *Leishmania sp.*, segregates in a dominant mendelian pattern, and a locus (termed *Ity*, *Bcg* or *Lsh*, respectively), identified on mouse chromosome 1, controls this susceptibility through an alteration in macrophage function (Lissner *et* al., 1983). It has been demonstrated that resident peritoneal macrophages from Ity^R mice exhibit a greater bactericidal capacity against *S. typhimurium* than those from Ity^S mice (Lissner *et* al., 1985). A gene designated *Nramp* (natural resistance associated macrophage protein) has been identified within the *Ity* locus and this gene, associated with susceptibility to *Salmonella* infection, encodes an integral membrane protein with structural similarity to transport proteins. It may function in macrophages to transport microbicidal substances into phagosomes (Vidal *et* al., 1996; Gruenheid *et* al., 1997; Govoni *et* al., 1999).

IVET and STM

Other sophisticated systems have been developed to study interactions of *S. enterica* with its host, which take advantage of the amenability of this bacterium to genetic manipulation and the fact that it has been extensively investigated (Gulig, 1996). These systems include mammalian cell culture systems that simulate intestinal epithelial cell or macrophage interactions with *S. enterica*. These allow *Salmonella* invasiveness to be investigated prior to *in vivo* testing for attenuation (Finlay and

Falkow, 1989; Galan and Curtiss, 1989; Galan and Sansonetti, 1996). Also, to select for genes that are wholly required for the survival of *S. enterica* during infection, some investigators (Mahan *et* al., 1993, 1994), used the mouse model to develop the new strategy of *in vivo* expression technology (IVET). More recently, signature-tagged mutagenesis (STM) has been developed as a genomic technique to determine whether a specific genetic change results in attenuated virulence in a host. This novel methodology allows investigators to test many different insertional mutants simultaneously in a living host (Shea and Holden, 2000).

1.4 INTERACTIONS OF *S. ENTERICA* WITH EPITHELIAL CELLS

1.4.1 Cellular mechanisms

The entrance of salmonellae into the T84 human colon epithelial cell line has been demonstrated to result in the production of D-myoinositol 1,4,5,6-tetrakisphosphate. This substance may promote chloride flux in the mammalian host and subsequent diarrhoea (Eckmann *et* al., 1997).

The primary sites of entry of *S. enterica* into the intestinal mucosa are the enterocytes (the columnar epithelial cells that line the intestine) and microfold cells (M cells), which are dome-like epithelial cells that cover Peyer's patches (Brandtzaeg, 1989; Finlay and Falkow, 1989; Siebers and Finlay, 1996). Peyer's patches consist of lymphoid follicles and form part of the gut-associated lymphoid tissue (GALT). In mice, M cells represent 5-10% of these cells. Electron microscope studies have demonstrated that *S. typhimurium* enters both M cells and cultured epithelial cells within 30 minutes of contact (Finlay and Falkow, 1990; Jones *et* al., 1994).

One mechanism of invasion involves typhoid bacilli being actively internalised by M cells and other organised lymphoid tissue of the gut. From here they are

transported into the underlying lymphoid cells from where they enter the systemic circulation. *S. typhi* has been demonstrated to attach to and invade murine M cells, but is rapidly cleared from the Peyer's patches without destruction of the M cells. In contrast, invasion of murine M cells by *S. typhimurium* is accompanied by destruction of M cells and subsequently sloughing of the epithelium (Weinstein *et* al., 1998). The invading bacteria do not seem to replicate to any extent within the M cells, but move through the cell.

In the second invasive mechanism, bacilli are internalised by enterocytes via a process termed bacterial-mediated endocytosis (BME). *S. typhimurium* stimulates these normally non-phagocytic cells to undergo significant cytoskeletal rearrangements similar to those induced by exposure of cells to growth factors, that are visualised as localised membrane ruffling around the bacteria (Finlay and Falkow, 1988, 1990; Francis *et* al., 1992, 1993; Galan *et* al., 1992a; Jones *et* al., 1993; Pace *et* al., 1993; Garcia-del Portillo and Finlay, 1994; Ginocchio *et* al., 1994). Salmonellae are then internalised within membrane-bound vacuoles through which organisms transcytose from the apical to the basolateral surface of cell, without destroying the enterocyte (Takeuchi, 1967; Finlay *et* al., 1988). Leung and Finlay (1991) have shown that the ability of *S. typhimurium* to replicate in cultured epithelial cells is essential for virulence. After a lag period of 5-6 hours, typhoid bacilli replicate in these vacuoles, with a generation time of approximately 40-50 minutes (Finlay and Falkow, 1989). In response to *S. typhimurium*, the intestinal epithelium promotes an intense inflammatory response, clinically expressed by enteritis. It consists largely of the migration of polymorphonuclear leukocytes (PMN) toward and ultimately across the epithelial monolayer into the intestinal lumen (Kumar *et* al., 1982).

The formation of host filamentous structures named *Salmonella*-induced filaments (sif) has been demonstrated, which correlates with the start of intracellular replication of *S. typhimurium* (Garcia-del Portillo *et* al., 1993). The 16-kb *sifA* is a *Salmonella*-specific gene required for the bacterial-promoted formation of filamentous structures in the lysosomal vacuoles of infected epithelial cells. It has

been suggested that these filaments may help deliver nutrients to the vacuole-bound *S. typhimurium*, contributing to the pathogenicity in the murine typhoid fever model (Garcia-del Portillo *et* al., 1993; Stein *et* al., 1996).

1.4.2 Molecular and genetic mechanisms

Role of fimbriae and flagella

Surface components including fimbriae and flagella have been investigated for their role in mediating entry into eucaryotic cells. However, the role of fimbriae and flagella in infection remains controversial and is dependent upon the serovar of *S. enterica* examined and possibly also, the nature of the experimental model used. Recent evidence suggests that fimbrial adhesins of *S. typhimurium* play a role during bacterial attachment and invasion of the intestinal mucosa *in vitro* and *in vivo* (Lockman and Curtiss, 1992; Baumler *et* al., 1996; 1996a; 1997). Attachment mediated by fimbrial adhesins appears to be important for invasion of cultured epithelial cell lines *in vitro* (Ernst *et* al., 1990; Baumler *et* al., 1996b). Previous studies have shown that inactivation of biosynthetic genes for type I fimbriae, LP fimbriae or PE fimbriae, attenuate *S. typhimurium* mouse virulence approximately five-fold (Lockman and Curtiss, 1992; Baumler *et* al., 1996, 1996a). Plasmid-encoded fimbriae found only in *Salmonella* have been shown to bind specifically to M cells in the intestine (Low *et* al., 1996; Baumler *et* al., 1997a).

More recent studies have demonstrated that different fimbriae are involved in serovar-specific virulence traits. Enteric bacteria possess multiple fimbriae, many of which play critical roles in attachment to epithelial cell surfaces, but for example *S. enteritidis* fimbriae (SEF14) are restricted only to *S. enteritidis* (Clouthier *et* al., 1993). It has been recently shown that SEF14 and SEF17 fimbriae mediate bacterial cell aggregation on inanimate surfaces (Woodward *et* al., 2000), and mutations that disrupt the *sef* operon decrease virulence in mice more than 1,000-fold (Edwards *et* al., 2000). Another example has been given more recently by Wilson and co-workers (2000), who expressed *S typhimurium* type I fimbriae within *S. enterica* serovars Gallinarum (*S. gallinarum*) and Pullorum (*S. pullorum*). Serovars Gallinarum and

Pullorum expressing *S. typhimurium* type I fimbriae exhibited 10- to 20-fold increased ability to adhere to and 20- to 60-fold increased invasion efficiency of the human epithelial Hep-2 cell line.

Recent experimental results have shown that the type IVB pili might be used by *S. enterica* serovar Typhi (*S. typhi*) as an intestinal cell adhesin, although a *pil*$^-$ mutant retained 5% to 25% of the adherence and/or invasion ability of the wild type strain, using a human intestinal epithelial cell line as a model (Zhang *et* al., 2000).

S. typhi entry into epithelial cells is dependent on intact flagella, as well as motility functions (Liu *et* al., 1988). Recent studies have shown that a non-flagellated (*fliC*) mutant of *S. enteritidis* was 50-fold less invasive for Caco-2 cells, than the wild type, whereas bacterial adherence remained unaffected (Van Asten *et* al., 2000). Of interest, combining a non-attenuating *fimA* mutation with a mutation in the flagellum synthesis, resulted in a 1,000-fold increase in attenuation of the *S. typhimurium* strain upon oral challenge in BALB/c mice (Lockman and Curtiss, 1992).

Motility is also linked with the chemotactic responses of the bacterium and chemotaxis may have a role to play in host cell entry (Khoramican-Falsafi *et* al., 1990; Jones *et* al., 1992), particularly if host cells are damaged (Uhlman and Jones, 1982). However, no difference in invasiveness of non-flagellate and non-motile flagellate *S. typhimurium* cells has been found (Khoramican-Falsafi *et* al., 1990). Also, *S. typhimurium* mutants lacking flagella or motility are as virulent as the wild type strain (Lockman and Curtiss, 1990).

Role of lipopolysaccharide (LPS)

LPS has been demonstrated to have a role in mediating the entry of organisms into epithelial cells in a serovar-dependent manner. Rough mutants (unable to form a complete LPS molecule) and wild type (smooth) *S. typhimurium*, both have the ability to invade cultured HeLa cells (Kilhlstran and Ebedo, 1976). In contrast, rough mutants of *S. typhi* and *S. choleraesuis* are both incapable of entry into epithelial cells and for *S. choleraesuis*, the LPS deficiency has been shown to affect

the ability of bacteria to transcytose through cells (Finlay *et* al., 1988; Mroczenski-Wildey *et* al., 1989).

In *S. enterica* and *E. coli*, the LPS molecule is divided into three distinct regions: (1) the hydrophobic membrane anchor designated lipid A, (2) a short chain of sugar residues with multiple phosphoryl substituents, referred to as the core oligosaccharide, and (3) the O antigen, a structurally diverse polymer composed of oligosaccharide repeats (Raetz, 1996). In *E. coli*, the *waaP* gene product has been recently shown to be responsible for phosphorylation of the first heptose residue of the LPS inner core region. WaaP was also shown to be necessary for the formation of a stable outer membrane (Yethon *et* al., 1998). A *S. typhimurium waaP* mutant has been recently demonstrated to elicit a rough phenotype, be sensitive to polymyxin and showed complete loss of virulence in mouse infection models (Yethon *et* al., 2000).

1.4.3 *Salmonella* pathogenicity islands (SPI)

Pathogenicity islands are chromosomal clusters of virulence genes found in pathogenic organisms, but absent or sporadically present in related nonpathogenic species (Groisman and Ochman, 2000). They are often found adjacent to tRNA genes, suggesting transfer into these broadly conserved sites, and contain atypical guanine and cytosine content from that of the rest of the chromosome. They are thought to be of extraneous origin (Groisman and Ochman, 2000).

SPI-1

SPI-1, a 40-kb segment, encodes virulence factors involved in invasion of intestinal epithelial cells, induction of neutrophil recruitment and secretion of intestinal fluid (Galan and Curtiss, 1989; Jones *et* al., 1994; McCormick *et* al., 1995; Watson *et* al., 1995; Galyov *et* al., 1997). SPI-1 is localised at centisome 63 on the *S. typhimurium* chromosome and contains 31 genes encoding the typical components of a type III secretion system, which plays a role in invasion (see below), the effector proteins secreted by this system, and the corresponding effector chaperones, transcriptional

regulatory factors, and a few proteins of unknown function (Galan, 1996; Groisman and Ochman, 2000). Indeed, the majority of genes encoded by *inv* (invasion), *spa* (surface presentation of antigens), and *prg* (PhoP repressed genes) operons, are believed to encode the structural components of the invasion-protein-export system (Klein *et* al., 2000). In mice, mutations in SPI-1 attenuate *S. enterica* serotype Typhimurium (*S. typhimurium*) between 15- to 50-fold after oral infection, but have no attenuating phenotype following intraperitoneal injection (Galan and Curtiss, 1989; Baumler *et* al., 1997). The Inv/Spa system has been also implicated in macrophage apoptosis *in vitro* (Groisman and Ochman, 2000).

Role of the invasion-protein-export system

Secreted or surface-exposed bacterial proteins have long been known to play central roles in bacterial-host interactions. In Gram-negative bacteria, these proteins must pass through both the inner membrane and the outer membrane. There are three types of protein secretion systems and all use ATP hydrolysis to drive secretion (MacBeth and Lee, 1993; Mecsas and Strauss, 1996). Proteins secreted by the type I system cross directly from the cytoplasm to the cell surface, by-passing the general secretory pathway completely. Type II secreted proteins use the general secretory pathway to reach the periplasm, where they make an intermediate stop, and then traverse the outer membrane through distinct channel proteins. Both type I and type II systems secrete proteins that are involved in pathogenesis. For example, α-hemolysin of *E. coli* uses a type I system, and pili of the enteropathogenic and enterotoxigenic *E. coli* use type II systems for export (Mecsas and Strauss, 1996). The multicomponent, highly conserved type III secretion system has been found in many Gram-negative bacteria that cause disease in humans and plants (Van Gijsegem *et* al., 1995; Galan, 1996; Galan and Sansonetti, 1996) and is responsible for transporting effector molecules directly from the cytoplasm to the cell surface, where they interact with mammalian cells and modify host cell proteins (Michiels *et* al., 1990). Type I and type III systems do not remove any part of the secreted protein (Mecsas and Strauss, 1996). In contrast to the type I and type II systems, type III secretion is triggered when a pathogen comes in close contact with host cells and hence, has been called contact-dependent secretion (Ginocchio *et* al., 1994; Rosqvist

et al., 1994; Watarai *et* al., 1995; Galan, 1996). Temperature, growth phase, and salt conditions are environmental cues known to induce synthesis of the secretion apparatus in various pathogens (Lee and Falkow, 1990; Hromockyj *et* al., 1992; Straley and Perry, 1995).

The biochemical functions of all effector proteins delivered by the Inv/Spa system are beginning to be elucidated (Groisman and Ochman, 2000). Recent findings suggest that genes from *S. typhimurium* SPI-1 are required for lethal infection, but are not essential for diarrhoea (Tsolis *et* al., 2000). One of the striking properties of the Inv/Spa system of *Salmonella* is its capacity to translocate effector proteins encoded outside the SPI-1 into the cell, as part of the signalling mechanism for bacterial internalisation (Kaniga *et* al., 1995; Hakansson *et* al., 1996). These include proteins of the *sip* locus (*Salmonella* invasion proteins), *sipABCD*, immediately downstream of the Inv/Spa secretion apparatus (Groisman and Ochman, 2000). Secreted effector proteins are also located on the *sop* locus (*Salmonella* outer proteins) and on the *invJ*, *spaO* and *sptP* genes (Hueck *et* al., 1995; Galan, 1996; Kaniga *et* al., 1996; Wood *et* al., 1996).

Mutations in some of the *S. typhimurium* genes, such as *invA*, *invC* and *invG*, result in cells which do not produce the invasion structure, namely the invasome, or do not secrete Sip proteins (Galan and Sansonetti, 1996). The *invH* gene located on SPI-1 encodes a non-fimbrial adhesin involved in adherence and disruption reduces both attachment to and invasion of cultured epithelial cells (Altmeyer *et* al., 1993; Boyd *et* al., 1997). In contrast, mutations in *invA* and *invE* encoded on SPI-1, render *S. enterica* serotypes non-invasive, but have no effect on adhesion to cultured epithelial cells (Galan and Curtiss, 1989; Ginocchio *et* al., 1992). The observation that SPI-1-mediated adhesion and invasion can be genetically separated, suggests that entry of *S. enterica* into epithelial cells is a two-step process. New results using a *ΔSPI-I* mutant (Murray and Lee, 2000) suggest that *hilA* is also required for bacterial colonisation of the host intestine.

Regulation of expression of the invasion system, including the type III secretion machinery and the invasome, appears complex. A number of different environmental factors, especially those associated with the intestine, such as oxygen tension, osmolarity, carbohydrate availability and bacterial growth phase, have been shown to influence the expression of the invasion genes in *S. typhimurium* (Ernst *et* al., 1990; Clark *et* al., 1998). SPI-1 encodes regulatory proteins governing the expression of genes located within the island, such as HilA, InvF, PhoP and SirA (Lee *et* al., 1992; Bajaj *et* al., 1995, 1996; Galan, 1996, Johnston *et* al., 1996). InvF is required for the optimal expression of several genes encoding SPI-1-secreted proteins (Darwin and Miller, 1999). HilA, a transcription factor encoded on SPI-1, has been shown to activate expression of many invasion genes encoded also on SPI-1 (Bajaj *et* al., 1995, 1996; Eichelberg and Galan, 1999). However, HilA expression is itself influenced by PhoP (Bajaj *et* al., 1995, 1996), and by a recently identified regulator, SirA (*Salmonella* invasion regulator; Johnston *et* al., 1996). How all these signals and regulators interact to affect the secretion apparatus and their substrates is unknown, but it has been proposed that the induction of *S. enterica* invasion proteins in response to multiple environmental cues, ensures that bacterial entry is limited to the specific sites where an invasion phenotype is required (Galan *et* al., 1992; Bajaj *et* al., 1995; Lucas *et* al., 2000).

SPI-2

Salmonella is unique among Gram-negative pathogens in that it harbours two distinct type III secretion systems, each contributing to distinct stages of the infectious process. This second type III system, designated Spi/Ssa and encoded within the SPI-2 pathogenicity island, differs from the Inv/Spa system in genetic organisation, phylogenetic distribution and function (Groisman and Ochman, 2000). The SPI-2 is a 40-kb segment located at centisome 30 on *Salmonella* chromosome (Ochman *et* al., 1996; Shea *et* al., 1996), immediately adjacent to the *valV* tRNA gene (Shea *et* al., 1996). The 32 genes constituting SPI-2, encode components of the Spi/Ssa secretion system, the putative effectors of the Spi/Ssa system, a two-component regulatory system (SpiR/SsrA) positively regulated by OmpR (Lee *et* al.,

2000), and several proteins whose functions are unknown (Groisman and Ochman, 2000).

The Spi/Ssa system is essential for causing systemic disease (Ochman *et* al., 1996; Shea *et* al., 1996). Specifically, the SPI-2 appears to enhance bacterial replication, rather than survival within host macrophages. One of the secreted proteins, SpiC, inhibits fusion of the *Salmonella*-containing phagosome with lysosomes (Groisman and Ochman, 2000). Nevertheless, *Salmonella* remains within a membrane-bound vacuole in both epithelial cells and macrophages.

Other *Salmonella* pathogenicity islands

Other *Salmonella* pathogenicity islands have been localised on the *Salmonella* chromosome. The SPI-3, a 17-kb segment that harbours 10 genes organised into 6 transcriptional units, has been identified at the *selC* tRNA gene locus (Blanc-Potard and Groisman, 1997). This includes the *mgtCB* operon encoding the macrophage survival protein MgtC required for virulence in mice, and the Mg^{2+} transporter MgtB, as well as a putative transcriptional regulator (SsrB) and proteins of unknown function (Groisman and Ochman, 2000). Recent studies have uncovered at least two additional pathogenicity islands in *Salmonella*. One of them, SPI-4, is a 27-kb region situated next to a putative tRNA gene at 92 minutes on the *Salmonella* chromosome map. One of the 18 genes within SPI-4 is known to be required for intramacrophage survival (Wong *et* al., 1998). A further pathogenicity island, SPI-5, is a 7.5-kb region that maps to the *serT* tRNA locus. Four, out of the six genes in this locus, have been implicated in cattle enteritis by *S. enterica* serovar Dublin (*S. dublin*). However, recent experiments suggest that SPI-5 does not play a role in systemic disease (Groisman and Ochman, 2000).

1.5 INTERACTIONS OF *S. ENTERICA* WITH MACROPHAGES

1.5.1 Cellular mechanisms

The ability of salmonellae to survive within macrophages is essential to pathogenesis and spread of the organisms into the systemic circulation. Studies on patients with typhoid fever and positive blood cultures have revealed that all the organisms are contained in the mononuclear cell fraction (Rubin *et* al., 1990). Eventually, organisms are taken up by tissue macrophages in the bone marrow, liver, spleen and Peyer's patches (Hornick *et* al., 1970; Rubin and Weinstein, 1977). During the asymptomatic incubation of typhoid fever, most organisms are localised intracellularly within macrophages and possibly epithelial cells (Looney and Steigbigel, 1986), but symptoms of typhoid fever occur only when a critical number of organisms have accumulated (Miller and Pegues, 2000).

Macrophages are able to ingest bacteria by phagocytosis because of the presence of membrane receptors for the Fc segment of antibody (IgG and IgM) and also for the components of the complement system (C1R and C3R). Using time-lapse light microscopic techniques, Alpuche-Aranda and collaborators (1994) proved that *S. typhimurium* cells do not always enter J774 macrophages by conventional phagocytosis. They demonstrated that salmonellae induce membrane ruffling in macrophages, a process similar to that observed in epithelial cells. This is followed by internalisation of bacteria into small 2- to 5-μm membrane-bound vacuoles, with a large amount of extracellular fluid, termed macropinosomes through fusion of the ends of membrane ruffles. After endocytosis, macropinosomes can fuse to form large vacuoles containing *Salmonella* cells that are termed spacious phagosomes (SP). The *Salmonella*-containing vacuole appears to fuse with the lysosomal compartment, although the phagosome may have delayed acidification and novel trafficking to the lysosomal compartment (Alpuche-Aranda *et* al., 1992; Oh *et* al., 1996; Rathman *et* al., 1997). The ability to induce internalisation by macrophages and epithelial cells could also protect *Salmonella* from phagocytosis by neutrophils.

Typhoid bacilli are rapidly killed by neutrophils, with less than 10% of an initial inoculum surviving after phagocytosis (Weiss *et* al., 1982).

S. enterica is cytotoxic to macrophages and this phenomenon has been correlated with expression of the specialised type III secretion system (Chen *et* al., 1996; Monack *et* al., 1996). Cytotoxicity has been believed to be the result of the induction of apoptosis in the macrophage, occurring between 20 minutes and 14 hours post-infection (Chen *et* al., 1996; Lindgren *et* al., 1996; Monack *et* al., 1996). More recently, Watson and co-workers (2000) proved that *Salmonella* strains are able to kill bovine alveolar macrophages by a mechanism distinct from apoptosis and that other types of macrophages may be killed by a similar, non-apoptotic mechanism. This has important implications for future studies of *Salmonella*-induced macrophage lysis.

Experiments with macrophages from outbred and inbred mice with different resistances to *S. typhimurium*, indicated that a host component was essential to SP formation. The result that SPs were formed or maintained more easily in susceptible mice suggested that SP formation may be a factor in host susceptibility to *Salmonella*. For example, Ity^S mice are defective in restricting the growth of intracellular pathogens within macrophages. In addition to *Ity*, other loci which contribute to mouse susceptibility to *S. typhimurium* have been identified. These include *Xid*, a locus within the major histocompatibility complex, which controls B lymphocyte function in CBA/HN mice (O'Brien *et* al., 1979), and Lps^d, a locus responsible for lipopolysaccharide hyporesponsiveness in C3H/HeJ mice (O'Brien *et* al., 1980).

Serotype-specific bacterial factors are necessary for disease production and outcome. For example, both opsonised and non-opsonised, invasive serovar Typhimurium can enter into spacious phagosomes of murine macrophages, replicate, and apparently utilise these host vehicles to disseminate via the lymphatic system (Alpuche-Aranda *et* al., 1995; Mills and Finlay, 1998). Conversely, non-opsonised serovar Typhi strains can enter and thrive in human macrophages, but these same

bacteria are killed more rapidly in murine macrophages (Alpuche-Aranda *et* al., 1995; Schwan and Kopecko, 1997). Studies suggest that non-opsonised Typhimurium enters, multiplies within, and causes death of macrophages, leading to a highly virulent infection and death of the mice within 14 days. In striking contrast, non-opsonised Typhi survives silently and chronically within human macrophages, causing little cell death, which allows intra-host dissemination and typhoid fever with low host mortality (Schwan *et* al., 2000).

1.5.2 Genetic and molecular mechanisms

Microbicidal mechanisms of phagocytes

The antimicrobial mechanisms of professional phagocytes have been classified as oxygen-dependent and oxygen-independent. The oxygen-dependent mechanisms generate reactive molecules, such as superoxide anion, hydrogen peroxide, hypochlorite molecules and hydroxyl radicals, on binding the bacteria to the host cell surface and also within the phagosome. Alternatively, the oxygen-independent mechanisms include the acidification of the phagosome to approximately pH 3.0, the activation of degradative enzymes dumped into the phagosome at the time of fusion with the lysosome, and the antibiotic actions of small cationic peptides called defensins, which have been discovered in polymorphonuclear leukocytes in a number of species (Karlsson *et* al., 1995). Only oxygen-independent killing mechanisms are required to kill salmonellae within neutrophils (Looney and Steigbigel, 1986) and resistance to oxygen-dependent killing mechanisms may be more important within macrophages (Miller and Pegues, 2000).

Recent studies (Burns-Keliher *et* al., 1998) focused on examination of different protein patterns exhibited by *S. typhimurium* during growth within three different cell types, including intestinal epithelial cells, macrophages and liver cells. It has been shown that *S. typhimurium* responds to these different cellular environments with specific patterns of protein synthesis unique to each cell type. 142 proteins were detected in epithelial cells, of which 58 appear to be unique to growth within this cell line. 413 proteins were observed within macrophages, of which 157 appear

to be unique, and 183 proteins were detected in liver cells, of which 91 appear to be unique. The greatest number of proteins was synthesised within the macrophage and a possible explanation for this result is that *de novo* synthesis of a larger set of proteins may be required for survival and multiplication within these cells than within the epithelial cell lines, which do not mount an actively antimicrobial response upon invasion by bacteria.

During intra-macrophage survival and replication, salmonellae respond dynamically to the particular stresses of the microenvironment through the expression and/or repression of groups of genes, each designed to confer a selective advantage. Such genes, which are selectively activated *in vivo*, encode virulence factors that enable typhoid bacilli to survive the clearance mechanisms of the host cells. The size of the *Salmonella* genome has been estimated at 4.9 x 10^6 bp and it encodes approximately 4,800 genes (Liu *et* al., 1993). Recently has been proved that mutations in about 4% of the *Salmonella* genome lead to 1,000-fold or greater attenuation in the mouse typhoid model of infection and most of these genes appear to be required during early stages of a natural infection (Bowe *et* al., 1998). The set of stresses within the macrophage and mechanisms employed by *S. enterica* to promote its survival will not be reviewed here as they do not form the subject of this project. However, the following section will focus mostly on the heat shock response and the proteins involved, which are the basis of this study.

1.6 IMMUNITY TO *S. ENTERICA* INFECTION

1.6.1 Introduction

The defence mechanisms appropriate for a particular bacterial infection are related to the structure of the invading bacteria, and hence the immunological mechanisms to which they are susceptible, and to the mechanism of their pathogenicity (Roitt *et* al., 1998). A characteristic of *S. enterica*, a facultative intracellular bacterium, is its

ability to survive, and even replicate, within phagocytes. Since this microorganism is able to find a niche where it is inaccessible to circulating antibodies, effective elimination requires immune mechanisms that are very different from the mechanisms of defence against extracellular bacteria. The following section will be focussed on the role of the different arms of the immune system in the control of *Salmonella* infection.

1.6.2 The use of a murine model to study *S. enterica* infection

The need to develop effective vaccines to the causative agent of typhoid fever, has stimulated the study of the immune responses to *S. enterica* infection. Most experiments with *S. enterica* infection employ *S. typhimurium*, which causes an invasive systemic disease in mice that resembles human typhoid fever (Collins, 1974). Genetically resistant mice can eliminate *S. typhimurium* through innate immune responses, but susceptible mice require both cell-mediated and humoral immunity, a characteristic of the responses generated by humans to typhoid fever (Collins, 1974; Hormaeche, 1979; Hormaeche *et* al., 1985; Mastroeni *et* al., 1993). Therefore, a murine model of the *S. enterica* infection is used, in which inbred mice strains that are genetically susceptible to *S. typhimurium* infection (Ity^S; discussed earlier in this chapter) are infected with low virulence strains of *S. typhimuirum*.

1.6.3 General immunologic principles

Any immune response involves, firstly, recognition of the pathogen, and secondly, mounting a reaction against it to eliminate it. Broadly speaking, the different types of immune response fall into two categories: innate (or non-adaptive) immune responses and adaptive immune responses. The important difference between these is that an adaptive immune response is highly specific for a particular pathogen (Roitt *et* al., 1998). Innate immunity consists of factors which are present prior to any contact with a particular microorganism and which are not enhanced by such exposure. Conversely, specific immunity is stimulated by the identification of specific macromolecules (antigens) and responses are enhanced, and defensive

capabilities increased, with each successive exposure to that antigen. Moreover, although the innate response does not alter on repeated exposure to a given infectious agent, the adaptive immune system "remembers" the infectious agent and can act to prevent it form causing disease later (Roitt *et* al., 1998). It is important, however, that both elements of immunity, although distinct, interact with each other.

The different stages of *S. typhimurium* infection are reflected in a variety of mechanisms of innate and acquired immunity that contribute to the response of this bacterium, and that differ in their importance during distinct infection stages (Makela and Hormaeche, 1997).

1.6.4 Innate immunity

The first line of defence against pathogenic bacteria consists of simple physicochemical barriers, such as the skin or mucous membranes, which prevent organisms gaining direct entry into the underlying tissues of the body (Finlay and Falkow, 1989a). In addition, humoral factors present in the blood and tissue fluid have anti-microbial activities. These include the complement system, a family of serum proteins activated in a proteolytic cascade, which can be triggered by the surface of the microorganism (Joiner *et* al., 1984). Complement proteins, such as C5-C9, can form a hydrophobic complex in the cell membrane of the bacterium, called a membrane attack complex, which results in the lysis of the bacterium by osmotic swelling (Joiner *et* al., 1984). Additionally, various components generated in the enzymatic cascade of the complement system, can themselves stimulate inflammatory processes (*e.g.* C5a and C3a), or act as chemoattractants (*e.g.* C5a) to draw phagocytic cells to the site of infection (Adams and Hamilton, 1984). Moreover, phagocytes have specific cell surface receptors for components of complement (*e.g.* C3b) and so, act as a mechanism to opsonise and phagocytose the bacterium (Adams and Hamilton 1984; Joiner *et* al., 1984).

1.6.5 Role of phagocytic cells in bacterial infection

The major factor in preventing microorganisms entering the body, is the involvement of professional phagocytes, namely mononuclear phagocytes (monocytes and macrophages) and polymorphonuclear leukocytes, such as neutrophils (Abbas *et* al., 1997; Roitt *et* al., 1998). Mononuclear phagocytes develop in the bone marrow, then migrate via the blood as monocytes, to organs such as liver, spleen and draining lymph nodes, where they mature into tissue-resident macrophages (Roitt *et* al., 1998). Such macrophages constitute the reticuloendothelial system (RES). The Kuppfer cells (liver-resident macrophages) are quantitatively the most important component of this system (Adams and Hamilton, 1984; Kaufmann, 1993). Although macrophages are a part of the innate immune system, they also play an important role in acting as, and generating effectors of specific immunity (Abbas *et* al., 1997). Neutrophils mature in the bone marrow and are released into the blood where they function for approximately 4-5 days. In contrast, mononuclear phagocytes have a much longer life span (Gulig, 1996).

Phagocytes respond rapidly to the invading bacteria, by chemotaxis through stimulation by inflammatory products (C5a) or components derived from bacteria, followed by target recognition, ingestion and then degradation (Abbas *et* al., 1997; Roitt *et* al., 1998). Phagocytosis involves the internalisation of the bacterial cell into a membrane-bound vesicle, the phagosome, and represents the first step in an endocytic pathway. The contents of the phagosome are destined to be destructed by fusion with azurophilic lysosomes. The anti-microbial activity of phagocytes is exerted before and after engulfment of the bacteria and involves two mechanisms: oxygen-independent and oxygen-dependent killing (Adams and Hamilton, 1984; Hasset and Cohen, 1989; Roitt *et* al., 1998).

Oxygen-independent mechanisms

These mechanisms may be more important than previously thought. Many bacteria can be killed by cells from patients with chronic granulomatous disease, whose macrophages lack a respiratory burst, or from patients with myeloperoxidase deficiency, which cannot produce hypohalous acids. Some of the bactericidal activities in these cases may be due to nitric oxide, but many microorganisms can also be killed anaerobically, so the oxygen-independent mechanisms have been extensively investigated (Roitt *et* al., 1998).The factors involved in oxygen-independent killing are very complex and they may all require phagolysosome fusion (Roitt *et* al., 1998). A large number of proteins and peptides are associated with the anti-microbial environment of the phagolysosome. For example, lysozyme is capable of breaking the peptidoglican layer of the bacterial cell wall and acid hydrolases manifest their degradative abilities. Also, lactoferrin can bind iron and render it unavailable to bacteria, even at an acid pH. Thus, the ability of polymorphs to kill some bacteria is lost if they are loaded with iron (Foster and Spector, 1995). Furthermore, there are a wider range of proteins with cytotoxic activities, which include azurocidin, cathepsin G, bactenencins, major basic protein (MBP), and bactericidal permeability increasing protein (BPI), but their mode of action is not yet understood (Lehrer *et* al., 1990). Cathepsin G and azurocidin are cationic proteins with a different pH optimum, both are related to elastase and have anti-bacterial properties, unrelated to their enzyme activity (Roitt *et* al., 1998).

Another important feature of the phagolysosome is the low pH. Following lysosome fusion, there is a transient rise in pH before acidification of the phagolysosome takes place and this occurs within 10-15 minutes (Roitt *et* al., 1998). The low pH (3.5-4.0) is created by the acidification of the phagolysosome using a Na^+/H^+ antiporter, and is inhibitory to a vast range of bacteria (Seguin *et* al., 1990, 1991; Foster, 1992; Slonczewski and Foster, 1996). Killing of microorganisms may be due to the acidification itself, though it is more likely to be related to the low pH optimum of lysosomal enzymes (Roitt *et* al., 1998).

Oxygen-dependent mechanisms

The oxygen-dependent killing mechanisms are initiated upon contact and engulfment of bacteria into the phagocyte, and are mediated via a respiratory burst associated with the hexose monophosphate shunt system. Lysosome fusion is not required for this pathway, and the reactions take place spontaneously during formation of the phagosome (Roitt *et* al., 1998). The principal enzyme involved in triggering the oxidative killing is NADPH-oxidase, an enzyme in the phagosome membrane, which reduces oxygen to superoxide anion (O_2^-) (Adams and Hamilton, 1984; Morel *et* al., 1991). This can then give rise to other reactive oxygen species (ROS), which are potentially toxic, such as hydroxyl radicals (OH), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). There is no detectable activity of NADPH-oxidase within unstimulated macrophages, but immunological evidence suggests that the enzyme is present in these cells (Morel *et* al., 1991).

Another important component of the oxygen-dependent killing mechanisms in phagocytes is nitric oxide (NO) (Vidal *et* al., 1993; Pacelli *et* al., 1995). NO is formed by the biotransformation of L-arginine into L-citrulline, by a nitric oxide synthase. NO is toxic for bacteria and its toxicity may be increased by interactions with products of the oxygen reduction pathway, such as H_2O_2, through formation of peroxynitrites and other toxic nitric oxide derived intermediates (Pacelli *et* al., 1995). If lysosome fusion occurs, myeloperoxidase may enter the phagosome. Myeloperoxidase or, under some circumstances, catalase from peroxisomes, acts on peroxides in the presence of halides to form additional toxic oxidants, such as hypohalite (HIO, HClO) (Roitt *et* al., 1998). For optimal expression of this mechanism, mouse macrophages need both activation by interferon gamma (IFN-γ), and triggering by tumour necrosis factor (TNF). Bacterial cells subjected to all these reactive oxygen species can be damaged at all fundamental levels, nucleic acids, lipids and proteins (Adams and Hamilton, 1984; Imlay and Linn, 1986).

Phagocytes ingest and attempt to destroy bacteria. However, at least to some degree, pathogenic intracellular bacteria, such as *S. enterica*, are resistant to degradation

within phagocytes. For effective control and eradication of *Salmonella*, acquired immunity is necessary (Mittrucker and Kaufmann, 2000).

The specific role of phagocytic cells

During the initial stages of infection by *S. typimurium*, both macrophages and neutrophilic granulocytes are critical for survival of the infected mice (Vassiloyanakopoulos *et* al., 1998). Macrophages phagocytose *S. typhimurium* and this process is enhanced by receptor-mediated uptake after opsonisation of salmonellae with IgG antibodies or complement (Mosser, 1994). However, by means of the specialised type III secretion system, bacterial proteins are injected into the host cells, allowing *S. typhimurium* to interfere with the signalling machinery. These interactions can force the uptake of *S. typhimurium* into macrophages, promote the intracellular survival of the pathogen, or cause macrophage apoptosis (Fields *et* al., 1986; Alpuche-Aranda *et* al., 1994; Monack *et* al., 1996).

Macrophage activation by cytokines, such as IFN-γ or TNF-α, appears to be a prerequisite for the destruction of *S. typhimurium*. Both cytokines are essential during the initial stages of *Salmonella* infection because they are involved in the induction of bactericidal mechanisms in macrophages (Gulig *et* al., 1997). These mechanisms include not only the production of reactive oxygen and nitrogen intermediates, but also improved handling of bacteria-containing phagosomes, rendering the bacteria accessible to lytic effector molecules from the lysosomes.

Overall, it is important to note that interactions between macrophages and *S. typhimurium* do not necessarily result in bacterial killing. This process is critically dependent on several factors, including: (1) the presence of functional Nramp1 molecules in macrophages, (2) the way *S. typhimurium* is engulfed by the macrophages, and (3) the activation state of the macrophage when it comes into contact with *S. typhimurium* (Mittrucker and Kaufmann, 2000).

Role of NK cells

Intracellular *Salmonella* also activates natural killer (NK) cells, either directly or by stimulating macrophage production of IL-12. NK cells produce IFN-γ, which in turn activates macrophages and promotes killing of phagocytosed bacteria. Thus, NK cells provide an early defence against salmonellae, prior to the development of specific immunity (Abbas *et* al., 1997).

Role of cytokines

During the initial stages of infection, cell wall components of *Salmonella*, such as lipopolysaccharide (LPS) and certain lipoproteins, induce a massive inflammatory response in the surrounding tissue, resulting in the expression of inflammatory cytokines (*e.g.* TNF-α, IL-1, IL-6, IL-12, IL-18), and a variety of chemokines that recruit cells of the immune system to these sites (Jung *et* al., 1995; Eckmann *et* al., 1996; McCormick *et* al., 1998). IFN-γ is also produced during early infection, as discussed earlier, with NK cells and macrophages as important sources at this stage (Schafer and Eisenstein, 1992; Ramarathinam *et* al., 1993). Macrophages and dendritic cells are major sources of IL-12 and IL-18, which induce production of IFN-γ, and in turn expression of IL-12 is enhanced by IFN-γ through a feedback loop (Trinchieri, 1995; Mastroeni *et* al., 1996, 1999).

1.6.6 Specific immunity

Although the innate mechanisms of the immune system are effective in restricting the initial growth of *S. typhimurium* for several days, these mechanisms fail to achieve sterile elimination of *Salmonella* from the host. Only the generation of a specific lymphocyte response allows their eventual effective eradication (Mittrucker and Kaufmann, 2000).

1.6.7 Role of T lymphocytes

Specific immunity is regulated by T lymphocytes, which identify antigens through a membrane-bound receptor, the T cell receptor (TCR) (Abbas *et* al., 1997). However, T cells require accessory cells to process and display the antigen. Such antigen presenting cells (APC) include cells such as monocytes, macrophages, Langerhans cells (specialised epithelial cells), and B cells (Abbas *et* al., 1997; Roitt *et* al., 1998). To some extent, many cell types can be stimulated to perform the function of antigen presentation during bacterial infection, including endothelial and epithelial cells (Abbas *et* al., 1997). This allows for activation and regulation of immune responses in almost any part of the host.

T cells are generally considered to mediate the effects of the immune system by producing soluble messengers called cytokines (Abbas *et* al., 1997; Roitt *et* al., 1998). These molecules modulate the activity of other cells, such as B cells or macrophages, and also enhance the activity of T cells. There is evidence that in early stages of bacterial infection cells of the immune system help dictate which type of specific immune response develops (Abbas *et* al., 1997).

There are a number of cell surface molecules, which are essential in determining the type of immune response generated during infection. These include molecules found on T cells, such as CD4 and CD8, and the major histocompatibility molecules I and II (MHCI and MHCII), which are found on APC (reviewed in Abbas *et* al., 1997; Roitt *et* al., 1998). T cells can only identify a processed antigen when it is associated with the products of MHCI or MHCII (Abbas *et* al., 1997) and this ability is restricted only to T cells expressing CD8 or CD4 on their surface, respectively.

1.6.8 The forms of antigens recognised by lymphocytes

Humoral responses and cell-mediated reactions are generally directed against different determinants on the antigens. Protein antigens do not induce antibody responses in the absence of T lymphocytes and they require the participation of helper T cells. For this reason, proteins are considered thymus-dependent antigens. Non-protein antigens, such as lipopolysaccharides and lipids, induce antibody responses without the requirement of helper T lymphocytes. Therefore they are called T-independent antigens (Abbas *et* al., 1997). Protein antigens are not presented by MHC molecules as intact proteins, but rather as processed peptides at the cell surface. The cells that process antigens are specialised APC, which are capable of stimulating T cell division (Roitt *et* al., 1998). The $CD4^+CD8^-$ and $CD4^-$ $CD8^+$ T cell subsets produce very different immune responses and this is largely dependent upon the type and source of the antigen.

The specificity of T lymphocytes for complexes of peptide antigens and MHC molecules determines several characteristics of T cell antigen recognition, which differ in fundamental ways from antigen recognition by antibody molecules. Most T lymphocytes recognise only peptides, whereas B cells can specifically recognise peptides, proteins, nucleic acids, polysaccharides, lipids and small chemicals (Abbas *et* al., 1997). Also, T cells recognise only linear determinants of peptides. In contrast, B cells may recognise conformational determinants that exist when proteins are in their native tertiary (folded) configuration or determinants that are exposed by denaturation or proteolysis. T cells recognise and respond to foreign peptide antigens only when the antigen is attached to the surfaces of other cells, whereas B cells and secreted antibodies bind soluble antigens in body fluids or cell surface antigens. This is because MHC molecules form part of the complex that T cells recognise (as reviewed in Abbas *et* al., 1997).

As mentioned previously, $CD4^+CD8^-$ T cells recognise peptides bound to class II MHC molecules. The major source of such peptides are extracellular proteins, including microbial proteins, which are endocytosed by APCs, and then enter the

acidic vesicular pathway that cells use to break down internalised proteins. In contrast, $CD4^-CD8^+$ T cells recognise peptides bound to class I MHC molecules. Such peptides are usually proteins found in the cytosol of APCs, including viral and tumoral proteins. Therefore, the association of antigens with class I or class II MHC molecules, is dependent upon whether the antigen is from an exogenous or endogenous source, and the trafficking of the antigens through different intracellular compartments (Brodsky and Guagliardi, 1991). The recognition of exogenous antigens by $CD4^+CD8^-$ helper T cells (T_H) results in cytokines being secreted that enhance the ability of phagocytes to kill bacteria or stimulate B cells to produce antibodies. In contrast, host cells expressing endogenous antigens associated with class I MHC molecules, are destroyed by $CD8^+CD4^-$ cytolitic T cells (CTL) that recognise this complex (Abbas *et* al., 1997).

1.6.9 Co-operation within the immune system

In order for an immune response to develop, antigen must come into contact with APCs, which in turn require cell to cell contact with T cells. Firstly, the immune system is organised into specialised lymphoid tissues into which many different types of cells, including B and T cells and APCs, can enter and reside (Abbas *et* al., 1997). This facilitates the close contact required for displaying antigens and for developing the required immune response. These lymphoid tissues include the spleen, liver, draining lymph nodes, and the Peyer's patches of the gut, and are connected by the blood and the lymph system. The antigen that enters into any part of the body, such as the skin or the intestine, is therefore likely to migrate to these tissues.

Secondly, antigens are structurally diverse and only a small population of B and T cells are likely to recognise specific portions of any particular antigen. These cells therefore migrate between tissues, a process called lymphocyte recirculation, such that the chances of antigen-specific lymphocytes coming into contact with the antigen are increased (Abbas *et* al., 1997). Thus, the organisation of the immune

system and the behaviour of the lymphoid cells act to produce a highly efficient monitoring system against infection.

1.6.10 Humoral immunity

Humoral immunity is mediated by antibodies produced by activated cells of the B lymphocyte lineage. B cells are formed in the bone marrow and possess membrane-bound antibody molecules which act as antigen-specific receptors (reviewed in Roitt *et* al., 1998).

Previously unstimulated B cells, which are exposed to antigens, generally produce IgM, which has multiple antigen-binding sites. This is called the primary response, whereas secondary responses are due to stimulation of expanded clones of memory B cells. Therefore, secondary responses develop more rapidly than primary responses, and larger amounts of antibodies are produced in the secondary responses. In general, a primary response to an antigen can be seen within 5-10 days after exposure to that antigen, and consists of IgM antibody. However, in the secondary response, the lag time in appearance of antibodies is greatly reduced and specific antibodies, mainly IgG, can be observed 1-3 days post-challenge. The production of different Ig isotypes enables humoral immune response to eliminate different types of microorganisms. Affinity maturation of antibodies makes an individual better able to combat persistent or recurrent infections and is also a reason why the optimal antigen doses required for secondary responses are lower than those for primary responses (Roitt *et* al., 1998).

1.6.11 The role of antibodies

Antibody has a number of roles in mediating protective immunity. It binds bacterial toxins and receptors (opsonisation) to prevent interaction with their respective targets, but also can activate the complement system via the classical pathway (Joiner *et* al., 1984). Macrophages and PMNs, in addition to having receptors for complement proteins (*e.g.* C1R and C3R), possess receptors for the carboxyl-

terminal region (F_C) of immunoglobulins, especially IgG_1 and IgG_3 in humans. Uptake of bacteria via these receptors is extremely efficient (Adams and Hamilton, 1984). Moreover, the binding of antibody or complement to these receptors stimulates the respiratory burst of phagocytes, increasing their anti-microbial activity (Joiner *et* al., 1984; Gulig, 1996).

1.6.12 Humoral immunity to *S. enterica* infection

Infection of mice with *S. typhimurium* results in a profound antibody response against both non-protein antigens, such as LPS, and protein antigens (Brown and Hormaeche, 1989). Vaccination of resistant mice with either attenuated or killed *Salmonella* induces protection against secondary infection with an otherwise lethal dose of virulent bacteria, and this protection could be passively transferred to naïve mice with serum (Eisenstein *et* al., 1984). It is known that people infected with *S. typhi*, or vaccine derivatives that have been attenuated, produce significant levels of IgM and IgG. Moreover, most of this is likely to be due to the polyclonal stimulation of B cells with LPS (Forrest *et* al., 1991; Tacket *et* al., 1992a). In susceptible mice, however, the situation is different. Killed bacterial vaccines generate only partial protection against challenge with virulent *Salmonella*, and protection cannot be transferred by serum alone (Eisenstein *et* al., 1984). Vaccination of susceptible mice with live vaccines induced protection against challenge with virulent bacteria (Killar and Eisenstein, 1985), but both serum and T cells are required for successfully transferred protection (Mastroeni *et* al., 1993).

Infection of B-cell deficient mice (from the inherently susceptible C57BL/6 background) confirmed that antibodies were dispensable for control of attenuated *Salmonella*. However, these mice were more susceptible to oral infection with wild-type bacteria during both primary and secondary responses (Izhar *et* al., 1990). When challenged intravenously with virulent *Salmonella*, vaccinated mice were more susceptible than wild-type control animals, although a fraction of B cell-deficient mice survived challenge.

These data demonstrate the involvement of antibody-independent mechanisms in the control of *S. typhimurium* (Mittrucker *et* al., 2000). Hence, antibodies participate in protection against *S. typhimurium*, and in resistant mice, antibodies alone are sufficient for control of virulent bacteria. In susceptible mice, where the infection with virulent *S. typhimurium* imposes a more stringent challenge on the immune system, antibodies participate in control, but protection depends on additional mechanisms (Mittrucker and Kaufmann, 2000).

Specific roles of antibodies

Antibodies could perform several functions during the different stages of *Salmonella* infection. In the intestinal lumen, antibodies (particularly IgM and IgA) could block penetration of salmonellae into deeper tissues. Therefore, the mucosal immune system is thought to provide an early line of defence against *Salmonella*. In the gut mucosa, there are sites that contain organised mucosal lymphoid tissue that sample lumenal antigens, resulting in the stimulation of both T cells and B lymphoblasts committed to IgA synthesis. This leads to secretion of IgA with specificity for the O-antigen of LPS (Slauch *et* al., 1993). This prevents the bacteria from contacting the mucosal surface by agglutination, and subsequently they are cleared by peristalsis (Mittrucker and Kaufmann, 2000). Indeed, injection of a B cell hybridoma producing *Salmonella*-specific IgA has been shown to prevent oral infection of mice (Michetti *et* al., 1992) by the inhibition of cellular adhesion and infection (Michetti *et* al., 1994).

After bacterial migration from the intestine into the Peyer's patches, mesenteric lymph nodes, and ultimately spleen and liver, antibodies could enhance bacterial engulfment and macrophage activation via F_C-receptor-mediated or complement-receptor-mediated phagocytosis (Armstrong and Hart, 1975; Mosser, 1994), although salmonellae seem to be insensitive to complement lysis directly. The presence of such mechanisms was revealed in experiments that analysed the kinetics of bacterial clearance from blood after systemic infection (Cheers and Ho, 1983; Saxen, 1984).

1.6.13 Cell-mediated immunity

Differentiation of $CD4^+$ T cells into T_H subsets

$CD4^+$ T cells (T_H) have two different profiles of cytokine production (T_H1 and T_H2) and these patterns select between the two basic types of response mediated by $CD4^+$ T helper cells. All $CD4^+$ T cells come from a common origin and upon interaction with the antigen, these cells produce IL-2 (a T cell growth factor) and then develop into a population called T_H0. T_H0 cells produce various mixtures of cytokines and, depending upon the source of antigen, type of APCs which present the antigen, and cytokines produced, the T_H0 cells subsequently develop into either a T_H1 or T_H2 subset (reviewed in Abbas *et* al., 1996) (see Figure 1.1).

The composition of the cytokines produced early on in the immune response determines which type of immune response develops. For example, both IL-12 and IFN-γ have been shown to promote a T_H1 type of immune response (Abbas *et* al., 1996). Macrophages that come in contact with bacteria secrete IL-12, which in turn causes T cells and NK cells (large granular lymphocytes) to produce IFN-γ. As previously described, IFN-γ activates macrophages and down-regulates T_H2 responses thereby helping polarise the immune responses towards the T_H1 type. In contrast, during helminth infection for example, IL-4, IL-10 and IL-13 are released and these cytokines help down-regulate a T_H1 immune response (Abbas *et* al., 1996).

T_H1-based immune responses are mainly cell-mediated, especially by macrophages (Roitt *et* al., 1998), therefore are particularly effective at eliminating bacteria, such as *S. enterica*. The antibodies produced in a T_H1 response generally act to assist cell-mediated immune responses (Abbas *et* al., 1996). In contrast, T_H2 cells encourage production of antibodies, especially IgE. T_H2 responses are associated with regulation of strong antibody and allergic responses (Roitt *et* al., 1998) and tend to be phagocyte-independent. Characteristically they show lack of ability to induce cell-mediated immunity, such as DTH (discussed later in this section). Cytokines from T_H1 cells inhibit the actions of T_H2 cells and *vice versa*. Such an antagonistic

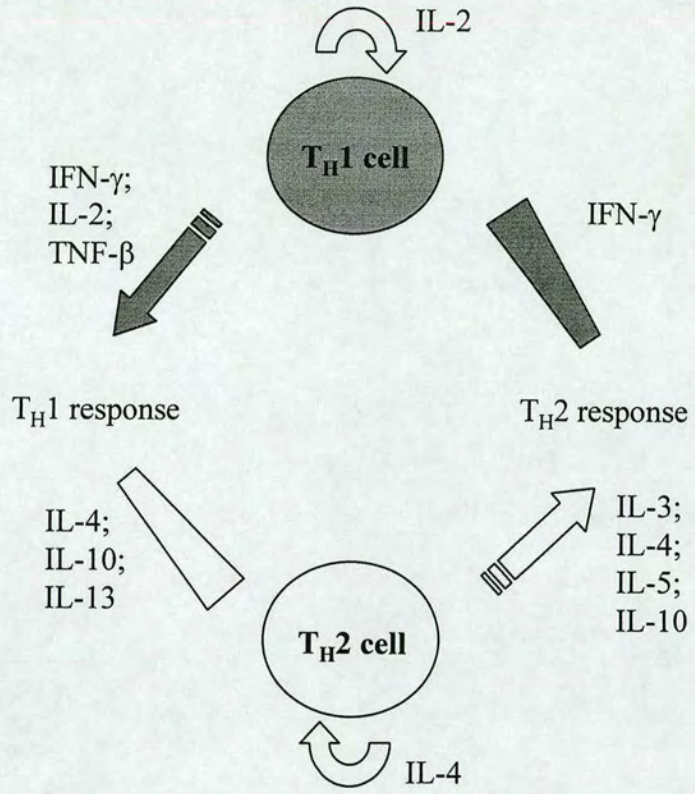

Figure 1.1

Effector functions of T_H1 and T_H2 cells.

The cytokines produced by T_H1 and T_H2 lymphocyte populations determine their opposing effector (pointed arrowheads) and inhibitory (blunt arrowheads) functions. The T_H1 and T_H2 pathways are symmetrical, each controlling a unique set of immune responses, and each augmenting the development of cells of the same subset, while suppressing the expansion and/or effector functions of the other subset. IL-2 and IL-4 are shown as autocrine growth factors for T_H1 and T_H2 cells, respectively (adapted from Abbas *et* al., 1996).

activity polarises the immune responses towards the most appropriate pathway for dealing with a particular pathogen.

The delayed-type hypersensitivity reaction (DTH)

The delayed-type hypersensitivity reaction (DTH) is a form of cell-mediated reaction in which the ultimate effector cell is the activated macrophage (Abbas *et* al., 1997), and also is an important method of investigating cell-mediated immunity against intracellular pathogens. Eradication of these organisms requires enhancement of the microbicidal function of phagocytes by T cell-derived cytokines. Purified protein derivative (PPD), a protein prepared from *M. tuberculosis*, will elicit a DTH response when injected into individuals who have recovered from primary tuberculosis or who have been vaccinated against tuberculosis. DTH reactions to most protein antigens may be transferred by antigen-sensitised $CD4^+$ T cells. *In vivo*, it is likely that DTH reactions elicited by phagocytosed bacteria, are predominantly mediated by $CD4^+$ T_H1 cells (Abbas *et* al., 1997).

1.6.14 Cell-mediated immunity to *S. enterica* infection

The role of T cells

A bulk of information on the role of T cells in *Salmonella* infection has accumulated since the first description of a DTH reaction after injection of *Salmonella* antigens into the footpad of *S. typhimurium*-infected mice (Collins and Mackaness, 1968). Experiments where T cell populations were depleted by antibodies or where T cell-enriched fractions of spleen cells were adaptively transferred, demonstrated that T cells are required for recovery from a primary infection with attenuated and virulent strains of *S. typhimurium*, and that T cells participate in protective immunity, which develops after vaccination with attenuated *Salmonella* strains (Chander *et* al., 1986; Nauciel, 1990; Mastroeni *et* al., 1992, 1993). Furthermore, nude mice or mice deficient in $\alpha\beta$T cells infected with attenuated strains of *S. typhimurium* fail to control infection and develop chronic disease or die (Hess *et* al., 1996; Sinha *et* al., 1997). Additionally, the generation of *Salmonella*-specific memory T cells at high

frequency at least in part explains the fast and efficient response that occurs in secondary *Salmonella* infection.

Besides the T cell-mediated mechanisms, there are other aspects of the immune response against *S. typhimurium* where T cells could have a regulatory or protective role. For example, it could easily be envisaged that T cells play a critical role in granuloma formation and in the containment of bacteria to local foci (Mittrucker and Kaufmann, 2000). T cells could regulate these processes either by direct cell-cell contact or by the secretion of lymphokines and chemokines.

The role of T cells and the importance of cytokines

The depletion of $CD4^+$ T cells, as opposed to $CD8^+$ T cells, was found to have a more pronounced effect on the control of primary *Salmonella* infection and on protection induced by vaccination with attenuated strains of *S. typhimurium*. Also, transfer of $CD4^+$ T cells from vaccinated mice into naïve recipients resulted in higher levels of protection than transfer of $CD8^+$ cells (Nauciel, 1990; Mastroeni *et* al., 1992; Pie *et* al., 1997). A large number of studies demonstrate that infection of mice with *Salmonella* induces a T_H1 response, characterised by the production of large amounts of cytokines associated with the T_H1 type of response, such as IL-12 and IFN-γ (Thatte *et* al., 1993; Eckmann *et* al., 1996; Pie *et* al., 1997).

The importance of IFN-γ and IL-12 was demonstrated by the observation that mouse mutants deficient in the IFN-γ receptor were highly susceptible to attenuated salmonellae (Hess *et* al., 1996). In addition, injection of resistant mice (CBA or A/J) with monoclonal antibodies specific to either IFN-γ or IL-12, resulted in these mice being overcome by the disease much more rapidly than a control group (Mastroeni *et* al., 1996). Moreover, addition of IFN-γ or IL-12 to susceptible mice (BALB/c) infected with *S. typhimurium* greatly enhanced their resistance to infection (Mastroeni *et* al., 1996). There are several reports indicating that neutralisation of IFN-γ is only critical during the initial phase of *Salmonella* infection (Muotiala and Makela, 1993; Pie *et* al., 1997). Furthermore, production of IFN-γ by T cells preceded the clearance of bacteria by several days (Pie *et* al., 1996, 1997).

Interestingly, it has been shown that IL-12 is critical for the induction of nitric oxide-mediated immunosuppression following attenuated *S. typhimurium* inoculation and, through its ability to stimulate IFN-γ production, can induce nitric oxide-producing suppressor macrophages (Schwacha and Eisenstein, 1997).

Because IL-12 plays a central role in both the induction and magnitude of a primary T_H1 response, recent studies have focussed on the use of native or recombinant IL-12 as an adjuvant in the administration of soluble protein antigens (Bliss *et* al., 1996; Mountford *et* al., 1996; Jankovic *et* al., 1997; Arulandam *et* al., 1999; Park *et* al., 2000; Stobie *et* al., 2000). This has led to the conclusion that IL-12 may be essential to sustain a sufficient number of memory and effector T_H1 cells to mediate long-term protection to an intracellular pathogen (Park *et* al., 2000; Stobie *et* al., 2000).

T_H1 cells can produce large amounts of TNF-α, which is crucial for immunity against *S. typhimurium* and *in vivo* neutralisation of TNF-α results in fatal infection (Mastroeni *et* al., 1991, 1992, 1993a; Gulig *et* al., 1997). It is believed to act synergistically with IFN-γ (Nakano *et* al., 1990; Ramarathinam *et* al., 1993; Berbanou and Nauciel, 1994). IL-10, which is produced by a variety of cells, including T cells, is able to counter-regulate both the production of other cytokines and macrophage activation, and a correlation between bacterial load and serum levels of IL-10 was observed in *Salmonella*-infected susceptible and resistant mice. However, neutralising anti-IL-10 antibodies did not modify the course of infection. It was therefore suggested that IL-10 is not involved in protection, but rather reflects severity of disease (Pie *et* al., 1996). Mice deficient in IL-4 production, a T_H2 cytokine, appeared to be more resistant to *S. typhimurium* than wild-type controls. They died later after infection with a wild-type strain of *S. typhimurium* and were less susceptible to infection with an attenuated strain. Furthermore, the mice failed to develop detectable lesions in the liver (Everest *et* al., 1997).

There is also evidence for participation of $CD8^+$ T cells in immunity to *S. typhimurium*. $CD8^+$ T cells can differentiate into cytolytic T cells (CTL), and antigen-specific target cell lysis is regarded as one of the main tasks of $CD8^+$ T cells

(Kaufmann, 1988). Because *S. typhimurium* is a facultative intracellular bacterium, it is possible that lysis of infected cells by CTLs would release bacteria from their protective habitat, rendering them accessible for activated phagocytes. Granules of CTLs contain granulysin, a protein that has been shown to express direct antibacterial activity against a wide range of bacteria, including *S. typhimurium* (Stenger *et* al., 1998). Moreover, depletion of $CD8^+$ T cells has been shown to reduce the ability to transfer protection against virulent *S. typhimurium* (Nauciel, 1990; Mastroeni *et* al., 1992). Also, it has been demonstrated that $CD8^+$ T cells can respond to bacterial structures that are conserved in different Gram-negative bacteria and presented by non-classical MHC class Ib molecules, such as Qa-1 (Lo *et* al., 1999).

Limited information is available on the role of γδT cells in immunity to *Salmonella* infection. Mice of a susceptible background and deficient in γδT cells were able to control systemic infection with an attenuated strain of *S. typhimurium* (Hess *et* al., 1996). However, αβT cells appear to be far more important for protective immunity against *Salmonella* infection (Mixter *et* al., 1994; Weintraub *et* al., 1997), but at sites where γδT cells represent a large fraction of T cells, such as the intestinal epithelium (Mixter *et* al., 1994), cells might gain some importance.

Host immuno-suppression mediated by *S. enterica*

In comparison to *Listeria monocytogenes*, eradication of *S. typhimurium* is rather a slow process. In normal mice, *L. monocytogenes* is eradicated between 7 and 10 days, whereas it takes around 6 weeks to eliminate even an attenuated strain of *S. typhimurium* (Hess *et* al., 1996; Mittrucker and Kaufmann, 2000). This difference can at least partly be explained by the observation that *S. typhimurium* causes immuno-suppression, partially mediated by NO, which is produced by macrophages during infection (MacFarlane *et* al., 1999). Although T cell activation occurs during the early stages of infection, it is possible that the lymphocyte response is blocked by this suppression and so bacterial eradication is delayed (Mittrucker and Kaufmann, 2000).

1.7 IMMUNISATION STRATEGIES AGAINST *S. ENTERICA*

1.7.1 Introduction

The following section will discuss the immunisation strategies developed against *S. enterica* infection and new directions towards elaborating new and effective vaccines against this pathogen.

1.7.2 Methods of vaccination against *Salmonella* infection

Three populations are at particularly high risk of developing typhoid fever and would benefit from immuno-prophylaxis with a vaccine. These include children in endemic areas (Ferreccio *et* al., 1984), travellers and military personnel from industrialised countries who visit endemic areas (Taylor *et* al., 1983), and clinical microbiology technicians (Blaser and Lofgren, 1981).

Passive protection by means of antiserum or immunoglobulin is not used to prevent typhoid or paratyphoid fever. The typhoid vaccines that have been evaluated in clinical trials and the few that have been used as licensed vaccine products can be grouped in the following categories: (1) inactivated whole-cell parenteral vaccines, (2) subunit parenteral vaccines, (3) attenuated *S. typhi* strains as live oral vaccines. Currently used and commercially available typhoid vaccines include: (1) heat-inactivated, phenol-preserved whole-cell parenteral vaccine (both liquid and lyophilised formulations and occasionally adsorbed to alum adjuvant), (2) acetone-inactivated and dried whole-cell parenteral vaccine (lyophilised formulation), (3) purified (non-denatured) Vi polysaccharide parenteral vaccine, (4) attenuated *galE*, Vi-negative strain live oral vaccine Ty21a.

1.7.3 Inactivated whole-cell parenteral vaccines

The acetone-killed whole-cell human typhoid vaccine given subcutaneously has been in use for many years because preservation of the Vi capsular antigen made the vaccine more immunogenic and protective than the previously used heat-phenol-killed vaccine that lacked the Vi antigen (Tacket and Levine, 1995). However, this acetone-killed whole-cell vaccine did not elicit good cell-mediated immunity, had limited protection, low efficacy and excessive reactogenicity (Levine *et* al., 1989). Inactivated (heat-killed, phenol-preserved) *S. typhi* was utilised as a parenteral vaccine as far back as 1896 by Pfeiffer and Kolle in Germany and Wright in Great Britain. Wright administered his vaccine to troops embarking for the Boer War in South Africa and by the World War I, typhoid vaccination became virtually routine in the British Army (Plotkin and Orenstein, 1999). This heat-inactivated phenol-preserved vaccine is by far the most widely available and utilised parenteral whole-cell vaccine world-wide. The heat-inactivated (phenol-preserved) vaccine must be maintained in the cold chain to maintain potency, but the lyophilised version retained potency even after 12 weeks of storage at high temperatures and was still antigenic after 5 years of storage at 4^0C (Plotkin and Orenstein, 1999).

With parenteral vaccines, the circulating antibody response is substantial and presumably provides the predominant protective effect. The most protective killed whole-cell parenteral vaccines stimulated the highest levels of H antibody, and a strain of *S. typhi* that lacked the H antigen failed to confer significant protection (Wahdan *et* al., 1975) after inoculation with parenteral inactivated whole-cell typhoid. The H antibody response is initially IgM and then becomes IgG (Kantele *et* al., 1991). These vaccines involve minimal mucosal secretory IgA antibody (Forrest *et* al., 1991; Nisini *et* al., 1993) and the cell-mediated response is not prominent (D'Amelio *et* al., 1988). However, effective immunity to *S. enterica* requires the development of a strong cell-mediated response and protection is short-lived with these vaccines (Collins, 1974; Chatfield *et* al., 1992).

1.7.4 Subunit parenteral vaccines

Many attempts were made to prepare extracts and sonicates of *S. typhi* and to purify antigens for use as parenteral vaccines. The various subunit immunising agents (which in the 1960s were referred to as "chemical vaccines") that have been evaluated for efficacy include: freeze and thaw extract vaccines, trypsinised extract vaccines and purified lipopolysaccharide (LPS O antigen) vaccines (hot water-phenol extraction method). Three major antigens have been identified: the capsular polysaccharide or Vi antigen, the O or somatic antigen (cell wall lipopolysaccharide) and the H or flagellar antigen. *S. typhi* is distinctive among the *Salmonella spp.* in possessing the Vi antigen composed of polygalacturonic acid, which is necessary for virulence (Hessel *et* al., 1999). The Vi antigen (named after its virulent properties in mice) physically prevents antibody binding to the O antigen and is also associated with inhibition of complement activation, lysis and phagocytosis (Robbins and Robbins, 1984; Looney and Steigbigel, 1986). Thus, the Vi antigen allows *S. typhi* to survive in the blood, leading to septicaemia. A report from a South African trial showed that over 3 years of follow-up, the single-dose Vi vaccine efficacy was 55% (Klugman *et* al., 1996) and depended solely on humoral immunity (Collins, 1974; Mastroeni *et* al., 1993). Purified Vi polysaccharide behaves like a T-lymphocyte-independent antigen and the serum antibody response is not boosted by administration of additional doses of Vi vaccine.

1.7.5 Attenuated *S. typhi* strains as live oral vaccines

Attenuated live bacteria have played a major role in recent vaccine development, as they not only are attenuated to the level where they do not produce the disease, but also they persist in the host long enough to stimulate protective immunity. Attenuated *Salmonella* generate long-term protection and their efficacy over dead vaccines in mice reflects their ability to elicit both an antibody response and cell-mediated immunity. The superior protection conferred in mice by live attenuated *S. typhimurium* vaccines correlates with a strong cellular T_{H1} type response coupled with high levels of anti-LPS IgG2a antibodies. A number of attenuated *S. typhi*

strains were evaluated in early phases of clinical trials, but were abandoned from further development.

Stocker and co-workers pioneered the use of auxotrophic mutants of *Salmonella* by transduction of an *aroA*::*Tn*10 lesion from the weakly pathogenic *S. typhimurium* LT2 into the fully virulent strain SL1344. The prototype *aroA* strains that also harboured deletion mutations in *purA*, were inhibited in their ability to proliferate, but remained viable. Hone and colleagues (1991) made precise deletion mutations in *aroC* and *aroD* in wild-type parent Ty2 (the wild type from which Ty21a was derived) resulting in the vaccine strain CVD908. This was the first engineered *S. typhi* vaccine that proved to be clinically well tolerated and highly immunogenic as a single-dose oral vaccine (Tacket *et* al., 1992a). CVD908 triggered strong cell-mediated immune responses, including cytokine production (particularly IFN-γ) (Sztein *et* al., 1994). Because *S. typhi* are intracellular pathogens, the cytotoxic lymphocytes (CTLs) may limit the progression of infection by destroying host cells harbouring the bacteria, a fact proved by *S. typhi* CVD908 immunised individuals who exhibited CTL effectors in blood, capable of killing B lymphocytes infected with the wild-type *S. typhi* (Sztein *et* al., 1995). Chatfield and co-workers observed that inactivation of *htrA*, a gene encoding a stress protein that functions as a serine protease, attenuates wild-type *S. typhimurium* in mice (Chatfield *et* al., 1992a). They further reported that mice immunised orally with *S. typhimurium* harbouring a deletion mutation in *htrA* were protected against challenge with a lethal dose of wild-type *S. typhimurium*. Also, it has been recently shown that *htrA* mutants of *S. typhimurium* are more susceptible i*n vitro* to oxidative stress than the wild-type, suggesting that the mutants may be less able to withstand oxidative killing within macrophages (Tacket *et* al., 2000). Based on these observations, an additional attenuating mutation was introduced into *htrA* of CVD908, resulting in strain CVD908-*htrA* that remains well tolerated and highly immunogenic (Levine *et* al., 1995; Tacket *et* al., 1997). This supports further development of CVD908-*htrA* as a single-dose vaccine against typhoid fever and a possible live vector for oral delivery of other vaccine antigens.

Moreover, the *S. typhi* vaccine strain CVD909 was created from CVD908-*htrA* (Virlogeux *et* al., 1996), resulting in a constitutive expression of the Vi antigen, even under high osmolarity conditions. After single intra-nasal immunisation with CVD909, the vaccine strain proved to be well tolerated in mice and elicited serum IgG anti-Vi antibodies in addition to other humoral, mucosal and cell-mediated immune responses observed after immunisation with CVD908-*htrA* (Wang *et* al., 2000). More extensive studies on CVD909 are in progress.

Curtiss and co-workers constructed the vaccine candidate strain X3927, a *cya*, *crp* double mutant of *S. typhi* strain Ty2 (Curtiss and Kelly, 1987; Curtiss *et* al., 1994). This was insufficiently attenuated to serve as a live oral vaccine in humans because subjects developed high temperatures and typhoid-like symptoms (Tacket *et* al., 1992). To achieve a greater degree of attenuation, it was introduced into X3927 a deletion mutation in *cdt*, a gene that affects the dissemination of *Salmonella* from gut-associated lymphoid tissue to deeper organs such as liver, spleen and bone marrow (Kelly *et* al., 1992). The resultant *cya*, *crp*, *cdt* triple mutant strain (X4073) was well tolerated given orally in single doses.

Hohmann and colleagues constructed two candidate *S. typhi* strains harbouring deletions in *phoP/phoQ*: strain Ty800, a derivative of Ty2 deleted in *phoP*, *phoQ*, which was well tolerated and immunogenic (Hohmann *et* al., 1996), and strain Ty445 which harbours an additional deletion in *aroA* and was overly attenuated and minimally immunogenic (Hohmann *et* al., 1996a). In a search for further attenuating lesions, investigators (Sydenham *et* al., 2000) employed the Tn*phoA* transposon to identify *S. typhimurium* derivations which are substantially attenuated in mice by the oral route of infection. One of these transposon mutants has an insert in the *surA* gene, a homologue that encodes a peptidyl-*cis*,*trans*-isomerase described initially in *E. coli*. The level of protection against challenge with the wild-type strain, following a single oral immunisation with the *surA* mutant, was comparable to levels reported for *aro* vaccines (Sydenham *et* al., 2000).

The attenuated live oral vaccine, which is currently available, is the *galE*-negative *S. typhi* strain Ty21a derived from the wild-type strain Ty2 by treatment with the mutagen N'-nitro-N-nitroso-guanidine (Germanier and Furer, 1975). Galactose residues are an important component of the smooth LPS O-antigen and the enzyme encoded by the *galE* gene, UDP-galactose-4-epimerase, isomerises UDP-glucose to UDP-galactose (and viceversa), providing galactose residues for incorporation into the O antigen. Ty21a exhibited complete loss of *galE* and lacked the Vi antigen. In the light of more recent data, it is recognised that the *galE* and Vi mutations in Ty21a do not explain the attenuation (Hone *et* al., 1988), but other mutations induced by chemical mutagenesis contributed to the diminished virulence. A mutation in *rpoS* (*katF*), which encodes an RNA polymerase sigma factor in particular, is thought to be important and this mutation diminishes the ability of the bacteria to survive a range of stress conditions (Robbe-Saule *et* al., 1995). Ty21a remains the most widely used vaccine at present and a liquid suspension formulation containing 2 to 10 x 10^9 c.f.u. of Ty21a has been licensed in several countries (Plotkin and Orenstein, 1999).

For live attenuated oral vaccines, the circulating antibody response is modest, but vigorous, intestinal secretory immunoglobulin A (sIgA) and cell-mediated immune responses are believed to be responsible for the protection conferred. The mucosal IgA and systemic cell-mediated immune responses appear to be directed toward the O and H antigens (Levine *et* al., 1989). The Vi is absent (Levine *et* al., 1990). The liquid formulation of Ty21a vaccine given in three doses conferred a high level of protection (96%) that persisted for the three years of surveillance (Wahdan *et* al., 1982). Three doses of vaccine in enteric-coated capsules given at an interval of every other day, conferred 62% protection over seven years of follow-up and with very little adverse reactions (Ivanoff *et* al., 1994).

<u>New recombinant *S. typhi* strains as live oral vaccines</u>

There is an ambitious need to apply recombinant DNA technology in order to engineer new *S. typhi* vaccine strains that will be as well tolerated as Ty21a, but much more immunogenic so that protective immunity can be elicited with a single

dose. Most research has focused on *S. typhimurium* because of its amenability to genetic manipulation and also because it produces a comparable typhoid-like disease in susceptible mice (although they do not develop gastro-enteritis and lack symptoms of diarrhoea). It has been shown that several genetic lesions that attenuate *S. typhimurium* in the murine model were effective in attenuating *S. typhi* in humans. Putative attenuated vaccine strains have been prepared by inactivating genes encoding various biochemical pathways, global regulatory systems, stress proteins, putative virulence properties, other regulatory genes.

1.7.6 Future vaccines

Subunit vaccines

For practical use, subunit vaccines are usually preferred over live vaccines, as live vaccines present a greater risk of complications. New vaccines based on the subunit principle have been developed, *e.g.* the hepatitis B surface protein vaccine. Nevertheless, live vaccines are believed to be more effective in inducing cell-mediated immunity because of protein synthesis by *S. enterica* in response to the host environment, and because of repeated antigen presentation (Kagaya *et* al., 1992). It is likely that a combination of proteins capable of stimulating T_H cells could be used as a protective vaccine. For example, the injection of *S. typhimurium*-derived porins or a hydrogen peroxide-inducible catalase (KatG) into mice, was shown to induce DTH and antibody responses and to provide protective immunity against a subsequent challenge with the virulent strain *S. typhimurium* LT2 (Udhayakumar and Muthukkaruppan, 1987; Kagaya *et* al., 1992).

Although they are less reactogenic, subunit vaccines have a tendency to be less immunogenic than the live vaccines (Dertzbaugh, 1998). To enhance the immunogenicity of an antigen, an adjuvant is essential to elicit an appropriate immune response. The adjuvant enhances deposition and persistence of the antigen, recruiting inflammatory cells and altering the balance between T_{H1} and T_{H2} type of immune response (Gupta and Siber, 1995; Vogel, 1995; Gupta *et* al., 1996). For example, vaccination with purified recombinant *Yersinia* Hsp60 induced antibody

responses, but only weak T-cell responses. However, when IL-12 was used as an adjuvant, purified Y-Hsp60 induced significant Y-Hsp60-specific T-cell responses and protection against subsequent challenge with *Yersiniae* (Noll and Autenrieth, 1996). Also, the incorporation of either IL-1β or TNF-α in aqueous or alum vaccine formulations enhanced antibody responses to a recombinant protein antigen (Rothel *et* al., 1998).

DNA vaccines

The discovery that plasmid DNA can be directly transfected into animals to elicit an immune response, has opened a new approach for producing vaccines (Dertzbaugh, 1998). Plasmid vectors used for DNA immunisation must have an origin of replication (*ori*) to permit transcription of plasmid DNA in a suitable prokaryotic host, the eukaryotic *ori* site is omitted to ensure that the plasmid does not persist in the vaccinee's cells and a selectable marker is included in the construct to help maintain the plasmid in the transformed prokaryotic host. The plasmid DNA expresses protection-inducing antigens and for high-level expression of the gene in the eukaryotic cells, the plasmid must possess an eukaryotic promoter upstream the gene. A transcriptional termination/polyadenylation signal sequence must also be located downstream of the gene (Ulmer *et* al., 1996; Dertzbaugh, 1998).

Genetic immunisation has been successfully used to elicit long-lasting humoral and cellular immune responses (Kumar and Sercarz, 1996) to a multitude of bacterial, viral and protozoan antigens (Donnelly *et* al., 1997). Indeed, when mice were injected with plasmid DNA encoding a single mycobacterial antigen (either the 65-kilodalton heat-shock protein Hsp65 or the major secreted mycobacterial antigen 85A), they developed specific cellular and humoral immune responses to the protein and proved protective against subsequent challenge with *Mycobacterium tuberculosis* (Huygen *et* al., 1996; Tascon *et* al., 1996; Lowrie *et* al., 1997; Lozes *et* al., 1997), whereas immunisation with the protein proved ineffective. It has been recently shown that vaccination with plasmid DNA encoding the mycobacterial antigen 85A generated particularly specific T_{H1} T-cell responses and $CD8^+$-mediated cytotoxicity (Denis *et* al., 1998) and that intramuscular DNA vaccination was the

method of choice (Tanghe *et* al., 2000). More recent attempts (Lefevre *et* al., 2000), using a plasmid encoding a 22-kDa protein normally present in the culture fluid of *M. tuberculosis*, proved ineffective after analysis of humoral and cell-mediated immunity, and of protection against intravenous challenge with *M. tuberculosis* H37Rv.

Because DNA vaccines produce the immunising material in the host, this is processed and presented by both class I and class II MHC molecules. The result is that DNA vaccines raise both cytolytic T-cells and antibodies. Part of the effectiveness of a "naked" DNA vaccine may be due to the immunostimulatory effect of the bacterial DNA, which differs in the relative frequency of CpG methylation compared to vertebrate DNA (Dertzbaugh, 1998; Sydenham *et* al., 2000). *S. typhi* OmpC porin has been tested as a DNA vaccine and elicited an antibody response, but CTL responses and further protection were not determined (Ulmer *et* al., 1996). There are several safety issues that must be considered concerning DNA vaccines, such as the ability of the plasmid to integrate into the genome of cell, to induce immunological tolerance and the risk of the plasmid inducing an auto-immune response to the host cell's DNA (Dertzbaugh, 1998).

1.8 *S. ENTERICA* COMPONENTS INVOLVED IN VIRULENCE AND IMMUNITY

1.8.1 Introduction

In the most general sense, any product that a bacterium synthesises, which enhances the growth or survival of a bacterium during its interaction with the host, can be considered a virulence factor and its corresponding coding sequence, a virulence gene (Mahan *et* al., 1996). Included in the broad category of virulence properties are adaptive responses to environmental stresses, which have been involved in the control of bacterial virulence gene expression. Groups of genes responding to given

environmental stresses can be considered virulence regulons even if their contribution to the infection process and pathogenesis remains relatively obscure. The expression of some virulence factors is activated shortly after introduction of the pathogen into the host, in order to maximise the chance of establishing a successful infection, while other non-virulence genes whose functions are inappropriate to this new environment, are quickly repressed (Mahan *et* al., 1996). Consequently, expression of certain virulence factors must be modulated in response not only to transition signals from the external source to the host, but also to other environmental signals encountered throughout the infection cycle. Pathogens encounter changes in temperature, osmolarity, oxygen tension, pH, and nutrient deprivation during infection. This section briefly describes *S. enterica* proteins involved in virulence and immunity and then is focussed mainly on GroEL structure, function, and its role in the immune reactions towards bacterial intracellular pathogens, such as *S. enterica*.

1.8.2 Environmental signals implicated in virulence gene expression

During the course of infection, *Salmonella* respond to a number of various signals, a process which allows its intracellular survival. For most pathogens to adapt and grow in the host, they must acquire iron. Thus iron plays a significant role in pathogenesis. Indeed, injection of iron compounds into the host has been shown to enhance the virulence of a number of bacterial pathogens, including *S. typhimurium* (Bullen and Griffiths, 1987). It has been proposed that polymorphonuclear leukocytes, in response to IL-1, release lactoferrin at the site of infection. Lactoferrin then binds iron at low pH, and subsequently it is taken up by macrophages, thus removing iron from the immediate environment (Mahan *et* al., 1996).

Salmonella has evolved a specialised iron-binding ligand, termed enterobactin, to acquire iron necessary for their multiplication (Neilands, 1993). Enterobactin, part of a chromosomally encoded system, competes with the host iron-binding proteins (transferrin and lactoferrin) to secure the iron. The role of enterobactin production in *S. typhimurium* virulence is controversial (Mahan *et* al., 1996), whereas *S. typhi*

strains defective in enterobactin synthesis were attenuated and showed a decreased ability to grow in cultured cells (Furman *et* al., 1994). Other virulence factors are also induced in response to low levels of iron, and regulation by iron acquisition in *Salmonella* is mediated by the Fur protein, an important virulence regulator (Stojiljkovic *et* al., 1994).

Invasiveness of *S. typhimurium* into cultured mammalian cells is repressed under conditions of high oxygen and induced under low oxygen (Schiemann and Shope, 1991). A regulatory screen designed to search for invasion mutants which invade aerobically (normally repressing conditions), has resulted in the isolation of the hyperinvasion locus (*hil*) on *S. typhimurium* chromosome (Lee *et* al., 1992). This region contained invasion genes (*invHFGEABC*) and *spa* (surface presentation antigen) (Galan and Curtiss, 1989).

Survival in acid may have clinical relevance, as enteric pathogens, such *S. enterica*, must pass through the stomach at a pH less than 3.0, for up to 2 hours before colonisation of the intestine. *S. typhimurium* harbours acid resistance mechanisms that contribute to its survival in the acid environment of the stomach and phagolysosome. The *S. typhimurium* PhoP/Q regulatory system is essential for full virulence (Miller *et* al., 1989) and is modulated by multiple environmental factors such as pH, phosphate, carbon, nitrogen, and oxygen (Behlau and Miller, 1993). PhoP/Q is required for (1) resistance to low pH (Fields *et* al., 1986), (2) invasion of mammalian cells (Behlau and Miller, 1993), (3) inhibition of macrophage vacuole acidification (pH 5.0) and spacious phagosome formation (Alpuche-Aranda *et* al., 1992), (4) resistance to antimicrobial peptides (Fields *et* al., 1989), (5) macrophage survival (Fields *et* al., 1989), (6) regulation of magnesium transport (Garcia-Vescovi *et* al., 1996), (7) resistance to the action of bile (Van Velkinburgh and Gunn, 1999), and (8) secretion of proteins by a type III mechanism (Pegues *et* al., 1995). It has been shown recently that the PhoP/Q system is responsible for regulating several modifications of the LPS, a mechanism that is likely to be necessary for *Salmonella* intracellular survival by providing resistance to the host innate immune system and by altering the immune recognition of this pathogen (Gunn *et* al., 2000).

Galan and Curtiss (1990) have showed that the ability of *S. typhimurium* to invade cultured epithelial cells was significantly impaired when the bacteria were grown in a low osmolarity medium. OmpR and EnvZ form a two-component system that regulates the major outer membrane porins, OmpF and OmpC, in response to medium osmolarity (for review, Slauch and Silhavy, 1996). Dorman and colleagues (1989) have showed that insertion mutations in *ompR* strongly attenuated virulence of *S. typhimurium* in a mouse model. Insertion mutations in *ompF* or *ompC* alone did not affect virulence. However, strains containing mutations in both *ompF* and *ompC* were attenuated, although the effect was not as severe as that seen in an *ompR* mutant (Chatfield *et* al., 1991). Pickard and co-workers (1994) have showed that synthesis and not export of the Vi antigen in *S. typhi* was affected by osmolarity and mutations in *ompR*.

Neutrophils and macrophages have several mechanisms to kill bacteria. These include the oxygen-dependent respiratory burst that generates superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Adams and Hamilton, 1984; Morel *et* al., 1991). Activated murine macrophages also produce nitric oxide (NO) (Vidal *et* al., 1993; Pacelli *et* al., 1995). Neutrophils from patients with chronic granulomatous disease are incapable of producing a respiratory burst. These patients have a high mortality rate from bacterial infections including *S. enterica*, suggesting that the respiratory burst is crucial in the defence against bacterial pathogens (Mahan *et* al., 1996).

S. typhimurium has regulons designed to deal with the cytotoxic products produced during the respiratory burst, with a large number of proteins being induced by OxyR or SoxRS in response to H_2O_2 or O_2^-, respectively (as reviewed in Demple, 1991). The OxyR protein activates transcription of approximately nine genes in *E. coli* in response to H_2O_2. These include *katG* encoding a HPI catalase, *dps* (DNA-binding protein from starved cells), and *ahpCF* encoding an NADPH-dependent alkyl hydroperoxidase. The latter protein will be discussed in more detail in the following sub-section of this chapter, as it has relevance to the experiments conducted in this study. The SoxRS regulon may also be important for survival in macrophages, but

its role in virulence may extend beyond protection against superoxide (Mahan *et* al., 1996). It has been shown that the SoxRS regulon is also activated in response to NO produced by activated murine macrophages (Nunoshiba *et* al., 1993). SoxR and SoxS control the expression of approximately ten genes in response to certain redox-cycling agents. These include transcription of *sodA* (superoxide dismutase), *nfo*, *micF* (Demple, 1991).

The alternative virulence factor RpoS regulates a variety of genes in response to starvation in *S. enterica*, and several of these genes could be considered as encoding virulence factors (as reviewed in Hengge-Aronis, 1996). In *S. typhimurium*, *rpoS* null strains are more sensitive to starvation, H_2O_2, acid stress, and DNA damage. These strains also show a 1,000-fold increase in oral 50% lethal dose compared to the parent strain (Fang *et* al., 1992).

Construction of strains carrying mutations in defined genes provides an important experimental strategy to analysing the role of individual proteins in bacterial virulence. Two groups of heat shock proteins have been studied extensively so far: DnaK and its functionally associated partners DnaJ and GrpE, and GroEL and its partner GroES. Failure to isolate null mutants in the *groE* genes suggest that these proteins are essential for survival (Fayet *et* al., 1989), and in the case of the DnaK group, although null mutants can be isolated at 30^0C, they have multiple severe growth defects (Paek and Walker, 1987). It is clear therefore that both of these heat shock protein groups perform important functions during normal growth at all temperatures. With regard to other heat shock proteins, it has been shown that mutations in the *htrA* gene result in attenuation of *S. typhimurium* (Chatfield *et* al., 1992b; Baumler *et* al., 1994).

Non-typhoid *Salmonella* strains that cause invasive diseases in humans and animals usually harbour a large (90-kb) virulence plasmid (Guiney *et* al., 1994). The primary function of the plasmid is to facilitate bacterial survival and proliferation in liver and spleen. The 8-kb plasmid region required for virulence is highly conserved and contains genes *spvABCD.* It has been shown that the *spv* operon in *S. dublin* was

significantly induced in stationary phase or in response to carbon starvation (Fang *et* al., 1991). Two regulatory factors play a crucial role in the control of *spv*. One is RpoS that governs the expression of various stationary phase-induced genes (Fang *et* al., 1992), and the second regulator is SpvR, which is encoded by the virulence plasmid and is regulated by RpoS (Spink *et* al., 1994).

1.8.3 Role of *Salmonella* components in immunity

Early studies on the antibody response to *Salmonella* in mice and humans were performed using immunoblots and ELISA (Brown and Hormaeche, 1989). Sera from mice infected with attenuated salmonellae, including an *aroA*$^-$ live vaccine strain, recognised up to 45 different bands on immunoblots at the height of the response, including lipoprotein, OmpA protein, porins, heat shock proteins and flagella. Adsorbtion of antisera with intact or sonicated smooth or rough salmonellae prior to immunoblotting showed that antibodies were directed against exposed, masked and intracellular antigens. The LPS response was much stronger in susceptible mice.

However, only a few *S. enterica*-derived components have been identified that can induce partial or full protective immunity against a virulent bacterial challenge. For example, when *S. typhimurium* outer membrane proteins (OMPs) were subcutaneously injected into BALB/c mice, 100% of the mice survived a lethal challenge and the protective immunity derived from the injection of the OMPs was maintained, with up to 70% of the immunised mice surviving the challenge after 6 months (Udhayakumar and Muthukkaruppan, 1987). Also, the injection of a *S. typhimurium*-derived hydrogen peroxide-inducible catalase (KatG) has been shown to induce varying degrees of protective immunity in mice (Kagaya *et* al., 1992). A number of studies have shown that various *S. typhimurium* proteins are recognised in a T_H1-regulated immune response. For example, DTH reactions have been elicited in mouse footpads following injections with proteins associated with pili and flagella (Gupta *et* al., 1996). KatE, the normal cellular catalase, was also shown to induce a DTH reaction in mice which were previously infected with *S. typhimurium*

yet, immunisation of mice with KatE failed to protect mice against a subsequent challenge with virulent organisms (Kagaya *et* al., 1992).

Early studies were focussed on the protective immunity against *S. typhimurium* infection in mice immunised with porins from mutant strains of *S. typhimurium* (Matsui and Arai, 1990). A high level of protection against infection was achieved in mice immunised with native porins from *S. typhimurium* LT2 (wild-type strain), but not from *S. typhimurium* mutant strains, which produce recombinant porins. However, antisera raised against these porins showed no significant differences in antibody titre against LT2 porin or LT2 whole cells. On the other hand, mice immunised with the native LT2 porin, but not recombinant or heat-denatured LT2 porin, exhibited significant levels of DTH reaction and IL-2 production when they were challenged with whole cells of *S. typhimurium* LT2. These findings suggest that the high level of protection induced by the native LT2 porin immunisation is dependent on the induction of cell-mediated immunity. Recent studies (Singh *et* al., 2000) that have investigated the antigenic specificity of the humoral immune response to infection by *S. typhimurium* revealed that immune sera from *S. typhimurium* LT2-immunised mice recognise OmpC and OmpD porins and the LPS (O antigen).

Fimbriae play a critical role in virulence by allowing bacteria to interact with host cells and other solid substrates. As mentioned earlier, it has been shown that inactivation of fimbriae-encoding genes attenuates *S. typhimurium* virulence (Lockman and Curtiss, 1992; Baumler *et* al., 1996, 1996a). Immunisation of mice with purified SEF14 fimbrial subunits induced a delayed-type hypersensitive response in mice to whole *S. enterica*, demonstrating that these fimbriae are expressed *in vivo* and stimulate cell-mediated immunity (Ogunniyi *et* al., 1994). Moreover, pretreatment of mice with anti-SEF14 antibodies has shown to protect mice from *S. enterica* infection (Peralta *et* al., 1994). However, other studies on the virulence of *sef* mutants have shown conflicting results (Thorns *et* al., 1996; Ogunniyi *et* al., 1997).

Since its initial description a decade ago, the surface display of heterologous peptides fused to the *Salmonella* flagellin has been extensively explored as a potential vaccine approach (Newton *et* al., 1989). Parenteral inoculation of live *Salmonella* cells or purified hybrid flagella/flagellin subunits, demonstrated the induction of antigen-specific immune responses (Wu *et* al., 1989; Stocker, 1991; McEwen *et* al., 1992; Newton *et* al., 1995; Verma *et* al., 1995a; Luna *et* al., 1996). Moreover, it has been demonstrated that *S. typhimurium* flagella (STF) are powerful monocytic activators and potent inducers of proinflammatory cytokine production.

The lipopolysaccharide (LPS or endotoxin) component of *S. typhimurium* has been initially correlated with the virulence of wild-type LT2 strain. It has been shown that mutants of LT2 with truncated polysaccharide portions of LPS exhibit reduced virulence. Moreover, polyclonal T cells and monoclonal T-cell hybridomas have been more reactive to heat-killed rough mutants than to heat-killed smooth strains, as measured by IL-2 production (Zirk *et* al., 1999). High concentrations of LPS associated with high bacteremia can lead to a severe form of enteric fever referred to as endotoxic shock. Many of the local and systemic pathophysiologic phenomena produced by LPS in the exposed host result from the ability of LPS to activate host inflammatory cells.

Of interest to the experiments performed over the course of this study is the *ahpCF* locus on *S. typhimurium* genome, which was found to contain two genes that encode the subunits of alkyl hydroperoxide reductase (Tartaglia *et* al., 1990). Ahp protects the cell membrane from lipid peroxidation during exposure to hydrogen peroxide or alkyl peroxides (such as cumene hydroperoxide) (Christman *et* al., 1985; Tartaglia *et* al., 1990). The *ahp* locus is known to be regulated by the transcriptional activator OxyR. Previous work in our laboratory has shown that the *ahp* operon of *S. typhimurium* is a macrophage-induced locus (Francis *et* al., 1997). Moreover, Taylor and co-workers (1998) in the laboratory showed that loss of *ahp* or *oxyR* did not significantly alter *S. typhimurium* SL1344 virulence in BALB/c mice. However, both cell-mediated and humoral immune responses to AhpC were found to develop during the course of infection, probably through type 1 T helper cells (T_H1)

activation. The presence of anti-AhpC antibodies from *S. typhimurium*, was detected at days 14 and 28 post-infection, indicating that this protein was recognised as part of the developing humoral response. It has been also shown that *S. typhimurium* derived AhpC protein induces significant DTH responses 33 and 104 days post-infection, suggesting that this polypeptide is recognised in the context of a T_H1 response.

1.8.4 Heat shock proteins and the immune response

Heat shock proteins (HSPs) occupy an important position in the complex web of host-parasite interactions. Interest in the antigenicity of HSPs was initially stimulated by reports that a highly immunoreactive 65-kDa protein of *Mycobacteria* had homology with a 60-kDa heat shock protein of *E. coli*. This protein was also shown to be related to a strongly immunoreactive antigen found in virtually all Gram-negative bacteria (Shinnick *et* al., 1988; Thole *et* al., 1988). It is now realised that HSPs play a role in the immune responses to many bacterial and parasitic pathogens, including the etiological agents of malaria, schistosomiasis, trypanosomiasis, filariasis, syphilis, tuberculosis, leprosy, legionnaires' disease, Lyme disease, and Q fever (Shinnick, 1991). From the perspective of the infected host, members of the highly conserved HSP protein families provide a major stimulus for humoral and cell-mediated immune responses, and HSPs may also have an important self-defence role in cells involved in the inflammatory response.

1.8.5 Factors that influence immunogenicity of HSPs

The immunoreactivity of HSPs raises questions as to why such highly conserved proteins should be the targets of an immune response. Possible explanations for this include that HSPs (1) are abundant cellular proteins, (2) have conserved epitopes that may prime the host for an immune response to these proteins, (3) may be preferentially processed for presentation due to either structural or functional features, and (4) may be virulence factors.

Abundance

During the growth of *E. coli* at 37^0C, Hsp60 and Hsp70 account for approximately 1.6% and 1.4% of total cell protein, respectively (Neidhardt *et* al., 1984). These proteins accumulate to even higher levels in bacteria undergoing stress. For example, Hsp70, Hsp60 and GroES can account for more than 15% of cell protein in heat-shocked cells (Neidhardt *et* al., 1984).

The synthesis and accumulation of subsets of HSPs can be stimulated by anoxia, amino acid or glucose deprivation, and exposure to H_2O_2, ethanol, or heavy metal ions. The stress exerted on internalised parasites by phagocytes may induce increased HSP synthesis in such pathogens in an attempt to protect themselves from host effector mechanisms. Indeed, even in the absence of a heat shock, two of the most abundantly expressed proteins of *S. typhimurium* following phagocytosis are Hsp70 and Hsp60 (Buchmeier and Heffron, 1990a). Moreover, mutants of *S. typhimurium* that do not increase the expression of stress response proteins upon phagocytosis are less able to survive within macrophages and less virulent *in vivo* than wild-type strain (Buchmeier and Heffron, 1990a).

Immunological priming

Since HSPs are highly conserved, infection by one pathogen might prime the host for an immune response against these proteins upon infection with a second pathogen. This suggests that the immune reactions should be directed primarily against conserved epitopes. However, this does not seem to be the case for antibody responses. Although antibodies directed against both conserved and non-conserved epitopes of Hsp60 are found following bacterial infections, the antibody response to parasite Hsp70 seems to be predominantly directed against non-conserved epitopes (Shinnick, 1991). On the other hand, T cells that recognise either conserved or non-conserved epitopes of the mycobacterial Hsp60 or Hsp70 homologues can be readily isolated (Shinnick, 1991).

Structural and functional considerations

Although most bacterial HSPs appear to be located intracellularly, some members of the Hsp70 and Hsp60 families appear to be surface accessible (Gillis *et* al., 1985; Shinnick, 1991; Ensgraber and Loos, 1992; Dunn *et* al., 1997). Also, certain eukaryotic cells undergoing heat shock or other stresses can express HSPs, including Hsp60, on their surfaces or even secrete them (Koga *et* al., 1989; Jarjour *et* al., 1989). Because of these features, HSPs can be efficiently processed and presented on the macrophage surface and, hence, become readily available for interaction with other components of the immune system. Two of the functions of HSPs may facilitate their processing and presentation. That is, members of the host Hsp70 family have been suggested to play roles in targeting intracellular proteins for lysosomal degradation and in antigen presentation on the macrophage cell surface (Chiang *et* al., 1989).

1.8.6 Members of HSP families as immunogens

Immunoreactive proteins have been identified that show homology with members of various HSP families.

The Hsp70 family

Perhaps the most highly conserved and abundant of the HSPs is the Hsp70 family (reviewed in Lindquist, 1986). The amino acid sequence of the human Hsp70 has 50% identity with that of the *E. coli* Hsp70, encoded by *dnaK* (Garsia *et* al., 1989). Members of the Hsp70 family have been shown to be antigens of several parasites and bacteria including *M. leprae*, *M. tuberculosis*, and *M. bovis* BCG. These 70-kDa HSPs react strongly with antibodies in sera from infected individuals and experimental animals. These proteins are potent T cell antigens that can elicit a DTH response in infected individuals, and can stimulate lymphocytes proliferation and IFN-γ production. Both conserved and non-conserved epitopes are immunoreactive (Pais *et* al, 1998). Hsp70 from *Mycobacterium, Chlamydia, Borrellia* and *Legionella spp.* have been recognised as common antigens in the immune responses to bacterial infection and in autoimmune diseases (Sampson *et* al., 1986; Anzola *et* al., 1992).

Recent studies have revealed that bacterial Hsp70 modulates immunity by directly inducing cytokine mRNA production in macrophages (Retzlaff *et* al., 1994).

1.8.7 The Hsp60 family

The GroEL protein (Hsp60) is the focus of this study. Before describing its involvement in the immune responses directed against the intracellular pathogen *Salmonella enterica*, it is useful to discuss briefly its structure and function.

Characterisation of GroEL at the genetic level

In *E. coli* and in many bacterial species, the 60-kDa GroEL protein is encoded in a bicistronic operon with the other heat-inducible protein, the 10-kDa GroES (Wong, 1992). *M. tuberculosis* is an exception and contains a gene encoding a second GroEL molecule (Shinnick *et* al., 1988).

Conservation of GroEL molecules

GroEL is highly conserved in both prokaryotes and eukaryotes. For example, the *S. typhi* GroEL is approximately 51% identical to that of the mouse mitochondrial homologue, and shares 75% identity with the *M. tuberculosis* homologue (see Figure 1.2 for an alignment). This homology can be easily explained in terms of the likely endosymbiotic origin of mitochondria and chloroplasts (Mayhew and Hartl, 1996).

Protein sequence analysis allowed Schmidt and collaborators (1992) to find considerable conservation, about 72 to 84% sequence similarity, between GroEL proteins from eight different eubacterial species. Also, there is a high 98.5% amino acid identity between GroEL molecules from *S. typhi* and *E. coli* (Lindler and Hayes, 1994). Very recent studies (Brocchieri and Karlin, 2000) have found that, after multiple alignment across 43 diverse Hsp60 sequences, the most conserved residues in terms of evolution, relate to the ATP/ADP and Mg^{2+} binding sites, and hydrophobic residues interacting with the substrate.

Figure 1.2

Alignment of different protein sequences of Hsp60 from various organisms.

The amino acid sequences of the Hsp60 proteins from different organisms are aligned. The alignment of the eukaryotic Hsp60 homologues shown represents the mature proteins, and nucleotides of the available sequence that encode this region (cds) are indicated next to the accession number, where appropriated. The sequences were obtained from the following accession numbers: mouse, X53584, cds 25-1665; rat kidney, X53585.em_ro, cds 1-1641; human, M34664.em_hum1, cds 25-1746; *E. coli*, P06139.swissprot; *S. typhi*, P48217.swissprot; *Y. enterocolitica*, D14078.em_ba; *M. tuberculosis*, P06806.swissprot. Abbreviations on the left side indicate the provenience of each amino acid strand. Key: mo, mouse; rki, rat kidney; h, human; ec, *E. coli*; st, *S. typhi*; ye, *Y. enterocolitica*; mt, *M. tuberculosis*.

Alignment of various GroEL sequences illustrates a tandemly repeated Gly-Gly-Met motif found at the carboxyl terminus. This is repeated three times for the *B. subtilis* GroEL and four times for both the *E. coli* and *C. burnetii* homologues (Li and Wong, 1992). Similar GGM motifs are also observed for most of the eukaryotic Hsp60 homologues.

Localisation of GroEL molecules within bacterial cells

While eubacterial GroEL and GroES are cytosolic proteins, homologues of GroEL and GroES are found in mitochondria and chloroplasts of eukaryotes (Mayhew and Hartl, 1996). None of the bacterial GroEL proteins possesses a leader sequence or other recognisable motifs that would indicate a secretory role (Craig *et* al., 1993). However, Ensgraber and Loos (1992) found that incubating *S. typhimurium* cells at 50^0C resulted in secretion of the 66-kDa Hsp into the supernatant. A growing number of reports indicate an extracytoplasmatic location for chaperonins (Vodkin and Williams, 1988; Scorpio *et* al., 1994), raising the possibility of unique mobilisation mechanisms specific for chaperonins and perhaps indicating novel biological functions for this highly conserved group of proteins. The recent description of chaperonin filaments (Trent *et* al., 1997) and a novel membrane-stabilising lipochaperonin activity (Torok *et* al., 1997) have expanded chaperonin function beyond protein folding and assembly. Surface-exposed Hsp60 has been reported in *Mycobacterium leprae* (Gillis *et* al., 1985), *S. typhimurium* (Ensgraber and Loos, 1992), and *Helicobacter pylori* (Dunn *et* al., 1997).

In *Legionella pneumophila*, Hsp60 is predominantly associated with the cell envelope and this bacterium has been demonstrated to have a novel mechanism, not present in *E. coli*, for transporting Hsp60 to the periplasm and for releasing it once the pathogen is within host cells (Garduno *et* al., 1998). In *Bordetella pertussis*, *Pseudomonas fluorescens*, and *Pseudomonas aeruginosa* surface exposure has been inferred by experiments in which whole cells were used to remove cross-reactive Hsp60 antibodies from a *L. pneumophila* Hsp60 antiserum (Plikaytis *et* al., 1987).

1.8.8 The structure of GroEL and GroES

The structures of the GroEL oligomer and its various complexes have been determined by X-ray crystallography (Boisvert *et* al., 1996) and by electron cryo-microscopy (Roseman *et* al., 1996). GroEL is a tetradecameric complex of identical 57-kDa subunits. The oligomer has a double toroidal structure revealed by electron microscopic analysis, is cylindrical and is formed from two heptameric rings stacked back-to-back. Each ring encloses a central cavity and there are holes in the sides of the structure (Saibil *et* al., 1993). The central cavity of the cylinder, the proposed site of polypeptide binding, is 4 to 6 nm in diameter and can expand considerably upon binding of the GroES cofactor.

GroES is a single heptameric ring of identical 10-kDa subunits (Chandrasekhar *et* al., 1986). The stoichiometry of the GroES 7-mer binding to the GroEL 14-mer, has been demonstrated to be 1:1 by both biochemical experiments and electron microscopy (Langer *et* al., 1992). The contact with GroEL is made through a flexible loop region in each GroES subunit (Landry *et* al., 1993) and occurs on only one side of the GroEL double toroid (Langer *et* al., 1992; Saibil *et* al., 1993). ATP binding induces a conformational change in the ring structure (Langer *et* al., 1992) and a change in the orientation of the outer domain with respect to the inner domain of each GroEL subunit (Saibil *et* al., 1993).

1.8.9 Functions of the GroEL-GroES chaperone team

GroEL and GroES are involved in folding of nascent chains, in the translation process itself (Gragerov *et* al., 1992; Horwich *et* al., 1993), in maintaining proteins destined to be secreted in a translocation-competent form (Kusukawa *et* al., 1989), and in refolding proteins after thermal damage (Ziemienowicz *et* al., 1993). In addition to their general role in protein folding, GroEL and GroES are involved in certain specific processes such as mutagenesis (Donnelly and Walker, 1989) and morphogenesis of some bacteriophage (Georgopoulos *et* al., 1972, 1973). Furthermore, these chaperones participate in general proteolysis (Straus *et* al., 1988).

Also, there is recent experimental evidence that indicates that GroEL interacts with lipid layers through its carboxyl terminus, increasing the molecular order of the layers (Erickson *et* al., 1987). Thus, it has been proposed that GroEL possesses a lipochaperonin activity that transiently stabilises membranes under stress. Recently, McLennan and Masters (1998) reported that GroEL is likely to have a role in the synthesis of diaminopimelic acid (DAP). They found that cells deprived of GroE have insufficient levels of DapA, the first enzyme in the DAP-synthesis, and so lyse because they fail to make cell walls.

It has been shown very recently that *E. coli* GroEL interacts strongly with a well-defined set of approximately 300 newly translated polypeptides, including essential components of the transcription/translation machinery and metabolic enzymes. About one third of these proteins are structurally unstable and repeatedly return to GroEL for conformational maintenance (Houry *et* al., 1999). Notably, proteins in their native states did not interact with GroEL. Evidence suggests that the preferred substrate of DnaK is an extended chain with hydrophobic residues, an early folding intermediate, whilst GroEL prefers a molten globule state that is a later intermediate (Landry *et* al., 1992). This suggests that DnaK and GroEL, at least in some cases, may act sequentially in the protein folding pathway.

Morphogenesis of bacteriophages

GroE proteins were originally identified as host genes necessary for bacteriophage T4 head assembly (Georgopoulos *et* al., 1972) and both GroEL and GroES are required for head assembly of phage λ (for review, see Friedman *et* al., 1984), tail assembly of T5 phages (Tilly and Georgopoulos, 1982), and both head and tail assembly of bacteriophage Mu (Grimaud and Toussaint, 1998).

1.8.10 The GroEL-GroES reaction cycle

In contrast to the actual mechanism of folding, the cooperation between GroEL and GroES, as well as the nucleotide exchange reactions that occur during a folding reaction (ATP hydrolysis) are well understood (Horovitz, 1998; Ranson *et* al., 1998).

Firstly, the unfolded polypeptide binds to the *trans* ring of GroEL, the ring distal to GroES in the asymmetric GroEL-GroES complex, thus forming a ternary *trans* complex. Secondly, the GroES-facilitated ATP binding at the distal ring to GroES, and the ATP hydrolysis in the GroES-bound ring, stimulate the release of GroES and then its rebinding to the polypeptide-bound ring, resulting in the encapsulation of the substrate in the *cis* cavity. In the presence of ATP, productive folding of the polypeptide substrate occurs in the cavity. Then ATP binding to the ring distal to GroES and also ATP hydrolysis in the GroES-bound ring, are followed by the release of the native polypeptide and GroES. Dissociation of ADP allows a new cycle to begin (Horovitz, 1998; Ranson *et* al., 1998).

1.8.11 Immunogenicity of GroEL and GroES

Unlike the case in many eubacteria (Hemmingsen *et* al., 1988), in eukaryotes Hsp60 homologues are not part of bicistronic operons, and GroES homologues have not been described (Hemmingsen *et* al., 1988).

An 10-kDa protein of *M. bovis* BCG reacts with antibodies and T cells elicited by *M. tuberculosis* and *M. bovis* (Minden *et* al., 1984), and a similarly sized antigen of *M. leprae* has been reported (Shinnick, 1991). Moreover, oro-gastric immunisation of mice with the GroES protein from *Helicobacter pylori* protected 80% of the animals against a subsequent challenge dose of 10^4 *H. felis.* The anti-GroES antibodies were predominantly IgG_1, suggesting T_H2 immunity (Ferrero *et* al., 1995). Very recently, Kimmel and colleagues (2000) have identified GroES as one of the 29 proteins from *H. pylori*, which strongly reacted with sera derived from *H. pylori*-infected patients. The Hsp10 antigen from *Chlamydia trachomatis* is recognised by a significant proportion of infected females and correlates with disease severity (Laverda *et* al., 2000). It should be also noted that *Coxiella burnettii* homologue does not appear to be antigenic (Vodkin and Williams, 1988).

Independent studies by Kaijser (1975) and Hoiby (1975) identified a 60-kDa protein as a cross-reactive antigen of *E. coli* and of *Pseudomonas aeruginosa.* This protein

was named "common antigen". Subsequent studies have shown that proteins cross-reactive with the common antigen are present in virtually all bacteria. Moreover, it was realised that the highly immunoreactive 65-kDa proteins of *M. tuberculosis* and *M. bovis* BCG are the mycobacterial counterparts of common antigen (Shinnick *et* al., 1988; Thole *et* al., 1988: Young *et* al., 1988).

The evidence that GroEL is a major immunodominant antigen comes initially from studies on *Mycobacteria*. The evidence includes that (1) antibodies and T cells that react with GroEL are easily isolated from sera of individuals infected with *M. tuberculosis* or *M. leprae* and (2) monoclonal antibodies to GroEL are frequently isolated from mice immunised with whole *Mycobacteria* or crude sonicates. An additional observation was that 20% of the *Mycobacteria*-reactive T cells in the *M. tuberculosis*-immunised mice reacted with GroEL (Kaufmann *et* al., 1987). The humoral and cellular immune responses to GroEL are directed against both conserved and non-conserved epitopes. T cells can be isolated from tuberculosis patients as well as from apparently uninfected individuals, which cross-react with GroEL from *E. coli*, *Mycobacteria spp.*, and humans (Munk *et* al., 1989; Lamb *et* al., 1989). Recent studies have revealed that bacterial GroEL can modulate immunity by directly inducing cytokine mRNA production in macrophages (Retzlaff *et* al., 1994).

Previous work in our laboratory has also indicated that GroEL is highly immunogenic. In BALB/c mice, immune responses to *S. enterica* infection are mainly controlled by $CD4^+$ T helper cells of the T_H1 subset (Thatte *et* al., 1993; Eckmann *et* al., 1996; Pie *et* al., 1997). The development of antibody responses to GroEL during the course of infection with *S. typhimurium* in mice, was determined by examining the serum from mice at 14 and 28 days post-infection. The antibody response to GroEL was of great magnitude and duration. Moreover, the *S. typhimurium*-derived GroEL has been shown to induce significant DTH responses in mouse footpads 33 and 104 days post-infection (Taylor, 1997).

Role of γδT cells

In the peripheral blood of healthy individuals, 1-10% of the T lymphocytes express the γδT cell receptor (TCR) (Kaufmann and Kabelitz, 1991). In general, γδT cells lack both the CD4 and the CD8 molecules on the surface, although some of them have been shown to express the CD8 or, to an even lesser extent, the CD4 molecule (Roitt *et* al., 1998). Interestingly, γδT cells represent the major T cell type in the epithelia of skin, small intestine, reproductive organs and respiratory tract of mice (Kaufmann and Kabelitz, 1991), and perform an important surveillance role at these locations. They exhibit non-specific killer activity, but also exhibit similar functional activities to αβT cells (Kaufmann and Kabelitz, 1991). γδT cells respond against infection by intracellular bacteria, such as *M. tuberculosis*, *Listeria monocytogenes*, *S. enterica* (Janis *et* al., 1989; Ohga *et* al., 1990; Emoto *et* al., 1992), and other pathogens.

That heat shock proteins represent antigens for γδT cells have been revealed by O'Brien and collaborators (1989) who found that many of their PPD-reactive γδT cell hybridomas were also stimulated by the mycobacterial Hsp60. It has been demonstrated that γδT cells contribute to antimicrobial immunity by recognising bacterial HSPs. Very importantly, HSP-reactive γδT cells may participate in autoimmune diseases. The isolation of a γδT cell clone with reactivity to HSPs from the synovial fluid of a rheumatoid arthritis patient may be taken as evidence of this (Holoshitz *et* al., 1989). Analysis of the specificity of T cells against GroEL using synthetic peptides revealed that many T cells recognise epitopes that are identical with or highly similar to the GroEL homologue in the host (Munk *et* al., 1989; Lamb *et* al., 1989). Such shared epitopes could induce autoimmune diseases under certain conditions.

The development of many autoimmune diseases has been etiologically linked to exposure to various infectious agents. For example, patients with a history of *Salmonella* infection have been shown to develop reactive arthritis. Lo and co-workers (2000) have indicated the involvement of MHC class Ib molecules in infection-induced autoimmune recognition and identified an immuno-dominant

epitope derived from the *S. typhimurium* GroEL molecule. This epitope was presented by the mouse MHC class Ib molecule Qa-1 and was recognised by $CD8^+$ cytotoxic T lymphocytes induced after natural infection. Elevated IgG and IgA antibodies to recombinant Hsp60 and stress proteins from *Mycobacteria spp.* have been reported in patients with reactive arthritis, systemic lupus erythematosus (SLE), and Crohn's disease (IgA class only) (Winfield and Jarjour, 1991). Handley and collaborators (1996) have reported that auto-antibodies to human Hsp60 in sera from patients with reactive arthritis, SLE, Reiter's syndrome and active tuberculosis, are best inhibited by *E. coli* GroEL, suggesting that exposure to normal environmental bacteria expressing GroEL, such as the normal gut flora, may be responsible for the induction of most human Hsp60 auto-antibodies. However, anti-human Hsp60 auto-antibodies were not indicative of the autoimmune disease in reactive arthritis and SLE, since they were found in similar titre in normal sera.

1.9 PHAGE DISPLAY SYSTEMS AS VECTORS USED IN IMMUNISATION

1.9.1 Introduction

Peptides can be used to raise epitope-specific antibodies, but in general such peptides are poor antigens *per se* and need to be coupled to a larger carrier or supplemented with an adjuvant. Many efforts have been made to circumvent these problems by encoding the peptide in a viral genome, in the hope that the peptide will be presented more effectively to the immune system as part of a coat protein in the assembled virion. Early studies in this direction have used the vaccinia virus, a large DNA virus, which has a wide host range, and may be able to stimulate both humoral and cell-mediated immunity (Moss and Flexner, 1987). It has been extensively used in humans as an element of a vaccination program to eradicate small pox. It is important to distinguish between vehicles, such as bacteria and viruses, which can propagate in the host organism, from vectors, such as plasmids or phages, which cannot (Hofnung and Charbit, 1993).

1.9.2 Bacteriophages as phage display systems

Bacteriophages offer obvious advantages over animal viruses for foreign epitope display. They are well understood at both the structural and genetic levels, and the viral particles themselves are highly immunogenic (Greenwood *et* al., 1991), therefore they could lead to cheap and effective vaccine development. In one such study a recombinant phage displaying a disease-specific protective B-cell epitope was used as a vaccine to confer protection against human respiratory syncytial virus infection in mice (Bastien *et* al., 1997). Also, phage particles displaying recombinant anti-idiotypic antibody ScFv (single-chain fragment-variable) polypeptides expressed on the phage have been used for maternal immunisation, protecting neo-natal mice against streptococcal infection (Magliani *et* al., 1998).

Greenwood and colleagues (1991) initially used filamentous bacteriophage fd to display small peptides of the major surface antigen from the malaria parasite, *Plasmodium falciparum*, as fusion proteins with the phage coat protein. Rabbits immunised with the hybrid fd bacteriophage developed specific antibodies against malaria epitopes in the fusion proteins and such antibodies predominated over antibodies directed against the wild-type coat protein itself. This proved that small peptides can elicit a strong and specific immune response. Subsequently, other investigators (Felici *et* al., 1993) used the filamentous bacteriophage fd for displaying *Bordetella pertussis* toxin epitopes, and immunising BALB/c mice. In this regard, the use of the phage library technology for vaccine development can be extended to any disease for which protective antibodies have been isolated, and especially to those cases where the epitope is discontinuous or has not been mapped (Felici *et* al., 1993).

More recent studies have used the M13 filamentous phage to express foreign gene products fused to the phage coat proteins. Antibodies carrying antigenic peptides grafted into their complementarity-determining region (CDR) loops at the immunoglobulin heavy-chain variable (V_H) region have been shown to be highly

immunogenic. With respect to this recent finding, Manoutcharian and collaborators (1999) have engineered a new type of immunogenic molecule by replacing all three CDR loops of the human immunoglobulin heavy-chain variable (V_H) domain with the *Taenia crassiceps* epitope PT1 displayed on the surface of M13 bacteriophage. When BALB/c mice were immunised with such phage particles, a strong protection against challenge infection in very susceptible hosts was obtained, with induction of a T_H1 response. Also, high levels of phage-specific antibodies were detected. The key advantage of this type of immunogen is that no adjuvant is required for its application and the proposed strategy for immunogen construction is potentially suitable for use in any host-pathogen interaction (Manoutcharian *et* al., 1999).

Different immunisation routes have been employed in immunisation protocols using recombinant M13. Filamentous M13 phage displaying single epitopes of the glycoprotein G (gG2) of HSV-2 have been used as immunogens via the subcutaneous route in BALB/c mice without any additional adjuvant (Grabowska *et* al., 2000). The antibody response that developed to gG2 was dependent on the dose of phage administered, and mice were protected against a subsequent lethal challenge. Because the ability to survive the harsh environment of the stomach in an antigenically intact form is an essential requirement for oral vaccines, epitope-displaying M13 bacteriophages have also been examined as vectors for an experimental oral vaccine (Zuercher *et* al., 2000). These bacteriophage induced anti-epitope antibodies, whereas oral immunisation with the purified bacteriophage coat-epitope fusion protein, induced anti-phage antibodies, but no anti-epitope antibodies. Therefore, there is evidence that epitope-displaying bacteriophages can be used to induce an epitope-specific antibody response via the oral route. All these considerations suggest a possible role for phage-displayed peptides as a means for inducing protective immunity against pathogens.

1.9.3 T7 bacteriophage

General characteristics

One of the original Type phages isolated independently by Demerec and Fano in 1940s, T7 is now considered the prototype of a group of virulent phages that are classified as the "T-like phages" within the *Podoviridae* family. These also include coliphage T3, *Salmonella* phage SP6, and *Klebsiella* phage K11. Members of this genus have double stranded DNA genomes, tailed polyhedral capsids and lytic replication cycle. They share characteristic features of virion morphology, genome organisation and replication strategy (Molineux, 1999).

T7 structure and assembly

T7 is an icosahedral phage with a capsid shell composed of 415 copies of the T7 capsid protein, encoded by gene 10. The capsid proteins are arranged as 60 hexamers on the faces of the shell and 11 pentamers at the vertices (Steven and Trus, 1986). Attached at the remaining vertex is the head-tail connector, encoded by gene 8, a short conical tail, encoded by genes 11 and 12, and 6 tail fibres, encoded by gene 17. Linear double stranded DNA is packaged into the capsid and has been extensively studied (Dunn and Studier, 1983; Steven and Trus, 1986). Phage assembly takes place inside the *E. coli* cell and mature phages are released by cell lysis.

T7 as a protein display system

T7 has been modified as a protein display system and is currently available as part of a kit from Novagen Inc. T7 has various properties that make it an attractive display vector. Unlike the filamentous systems, peptides or proteins displayed on the surface of T7 do not need to be capable of secretion through the cell membrane, a necessary step in filamentous phage assembly (Russel, 1991). It is very easy to grow, and replicates more rapidly than filamentous phages. Plaques form within 3 hours at 37^0C and cultures lyse 1-2 hours after infection. The T7 phage is extremely robust and is stable to many harsh conditions that inactivate other phage. The phage maintains infectivity after treatment with 1% SDS, 4M urea or 2M guanidinium

chloride, 10 mM EDTA, 0.1 M dithiothreitol (DTT), and alkaline conditions (pH range from 4.0 to 10.0). The phage is not stable at low pH (below pH 4.0) (Molineux, 1999). T7 is an excellent general cloning vector. Purified DNA is easy to obtain in large amounts, a high efficiency *in vitro* packaging system is available and the DNA is completely sequenced (39,937 bp).

The capsid protein is normally made in two forms, 10A (344 amino acids) and 10B (397 amino acids). 10B is produced by a translational frameshift at amino acid 341 of 10A, and makes about 10% of the capsid protein (Condron *et* al., 1991). However, functional capsids can be composed entirely of either 10A or 10B, or of various ratios of the proteins (Novagen Inc., USA). This finding provided the initial suggestion that the T7 capsid shell could accommodate variation, and that the region of the capsid protein unique to 10B might be on the surface of the phage and could be used for phage display. The frameshifted C-terminal extension of 10B protein is exposed on the outer faces of the icosahedral capsid, but it is not essential for phage growth. Cloning sites have been introduced at the 3' end of the modified gene 10B. Foreign carboxyl terminal extensions as long as 1,200 residues may replace the natural carboxyl terminal extension of 10B, and use of a complementing plasmid that supplies 10A is required for phage assembly. Therefore, foreign proteins are displayed on the phage surface at an average copy number of 0.1 to 1.0 per phage particle. Note that the large recombinant 10B protein presents a problem for phage expression/packaging. For short peptides, different T7 strains are available, which express a larger copy number of the recombinant 10B protein.

1.9.4 Project aims

It is well known that bacterial GroEL, which belongs to the major Hsp60 family, displays a high degree of genotypic and phenotypic homology among various microorganisms, and is an immunodominant antigen able to elicit a strong humoral and cellular immune response (Peetermans *et* al., 1995). GroEL has frequently been found to be an immunodominant molecule of intracellular pathogens, such as *S. enterica*. Previous studies have used purified GroEL molecules as immunising

agents and it has been found that they were able to induce antibody and cell-mediated immune responses along with protective immunity against a subsequent challenge with the original infectious agent from which they were isolated (Noll and Autenrieth, 1996). Also, it has been found that, when mycobacterial GroEL was conjugated with synthetic peptides and administered to mice in the absence of any adjuvant, high titres of anti-peptide IgG antibodies were induced in mice previously primed with live *M. bovis* BCG (Lussow *et* al., 1991). There is evidence that GroEL covalently linked to antigens are potent agents enhancing serum antibodies and $CD4^+$ T cell proliferative responses when administered by the systemic route, as well as secretory IgA and IgG antibodies given by the mucosal route. Therefore, bacterial GroEL molecules may function as natural adjuvants generating β-chemokines in the host (Lehner *et* al., 2000).

GroEL from *S. typhimurium* also has been found to be immunogenic in previous studies performed in our laboratory. The development of antibody responses to GroEL during the course of infection of mice with *S. typhimurium*, was determined by examining the serum at 14 and 28 days post-infection. The antibody response to GroEL was of great magnitude and duration, with substantial antibodies present even after 14 days. Moreover, the *S. typhimurium*-derived GroEL has been shown to induce significant DTH responses in mouse footpads 33 and 104 days post-infection, suggesting that this protein is recognised in the context of a T_H1 response (Taylor, 1997).

Early studies (Inchley, 1969; Inchley and Howard, 1969) showed that bacteriophage T4, when administered to mice without any adjuvant, was rapidly cleared from the circulation and was capable of inducing an antibody response. The initial exponential phase of the normal antibody response to T4 was completed by 7-10 days after immunisation and thereafter there was a relatively little increase in titre, with the antibody level maintained for up to 5-10 weeks in a dose-dependent manner.

Later, bacteriophages were used as expression vectors in which foreign gene products were fused to coat proteins and displayed on the surface of the phage particles. Subsequently, the high immunogenicity of these recombinant bacteriophages has been proved in different animal systems and production of antibodies against the foreign peptide overexpressed onto the phage capsid has been reported. Using such an approach, one of the objectives of this study is to clone the *groEL* fragment from *S. typhimurium* into bacteriophage T7 genome in such a way that the protein is expressed on the surface of the phage as a hybrid to coat protein 10B. The phage is then infected into an *E. coli* strain in order to amplify the hybrid phage. Subsequently, the purified recombinant phage expressing the *S. typhimurium* GroEL on its surface is used as an immunising agent in mice, without the co-administration of any adjuvant, to raise antibodies against GroEL from *S. typhimurium*. Using this strategy, the T7 bacteriophage is studied for its potential as a vector for immunisation against *S. enterica* infection in a mouse model. As part of this process, the sequence of *S. typhimurium groEL* gene was undertaken.

CHAPTER 2

Materials and methods

2.1 MATERIALS

2.1.1 Enzymes, chemicals and DNA manipulations

Chemicals and enzymes were purchased from a range of suppliers. Restriction enzymes were used according to manufacturers' instructions. DNA manipulations were carried out as described by Sambrook and co-workers (1989) unless otherwise stated. Rabbit anti-GroEL antibodies were supplied by Sigma, UK. Anti-mouse, anti-rabbit and anti-goat antibodies were provided by SAPU (Lanarkshire, UK). Stabilised solutions of [α-^{32}P]-dCTP (3,000 Ci/mmol) and [^{35}S]-methionine (1,175 Ci/mmol) were purchased from Amersham International Plc. (Buckinghamshire, UK), and ICN Pharmaceuticals Inc. (California, US), respectively. Synthetic oligonucleotides were purchased either from PE-Applied Biosystems (Cheshire, UK) or Sigma – Genosys Ltd. (Cambridgeshire, UK) and are listed in Table. 2.1.

Plasmids and bacterial strains and also their sources are shown in Tables 2.2 and 2.3.

Table 2.1 - Oligonucleotides used during the course of the present study.

Numbers shown refer to the position at which each of the primers lay with respect to the published sequence; the codes written in parentheses represent GenBank accession numbers; *ahpCF* (J05478), Tartaglia *et* al., 1990; *groEL* (U01039) from *S. typhi*, Lindler and Hayes, 1994; *groESL* (X07850) from *E. coli*, Hemmingsen *et* al., 1988; genome of bacteriophage T7 (V01146), Dunn and Studier, 1983.

Name	Oligonucleotide sequence	Comments and usage
GroEL53 1-18 (U01039)	5'-CCGAATTCGATGGCAGCTAAAGACGTA-3' *EcoR*I	PCR of the *S. typhimurium groEL* fragment (5'-3')
GroH3TTA 2117-2102 (X07850)	5'-TCACAAGCTTACATCATGCCGCCC-3' *Hin*dIII	PCR of the *S. typhimurium groEL* fragment (3'-5')
GroEL1 893-910 (X07850)	5'- GTACCGTGATCTCTGAAG-3'	Sequencing of the *S. typhimurium groEL* fragment (5'-3')
GroEC17131 1062-1044 (X07850)	5'-TCAATCTGCTGACGGATC-3'	Sequencing of the *S. typhimurium groEL* fragment (3'-5')
GroELrev 222-242 (X07850)	5'-GCTTTAGAGGCAACTTCTTTC-3'	Sequencing of the *S. typhimurium groEL* fragment (3'-5')
T7SelectUP 21457-21476 (V01146)	5'-GGAGCTGTCGTATTCCAGTC-3'	PCR of the region surrounding the multiple cloning site in T7Select1-1 System (5'-3')
T7Select DOWN 21563-21582 (V01146)	5'-TTGGGGAGTTCTGGGCAAAT-3'	PCR of the region surrounding the multiple cloning site in T7Select1-1 System (3'-5')
SPHI-ANTI739 503-479 (J05478)	5'-ACATGCATGCTGTCGAAGTTACGG-3'	Sequencing of the *S. typhimurium ahpC* fragment (3'-5')

Table 2.2 Table showing all the plasmids and their derivatives mentioned in this thesis.

Shown is a list of plasmids and their derivatives used in this study. Codes written in parentheses are GenBank accession numbers.

Plasmid	Relevant features	Source/ Reference
pBR325 (L08855)	pBR322 derivative, carries the Cml^R gene from phage P1 in which the unique *Eco*RI site is located. Amp^R, Tet^R, Cml^R	Bolivar, 1978; Blank and Wilson, 1982
pIB1	pBR325 derivative containing an *Eco*RI/*Hin*dIII *groEL* fragment of approximately 1.6 kb, subcloned from *S. typhimurium* SL1344 chromosome by PCR; Amp^R, Tet^S, Cml^S	This study (Figure 3.4)
pAR5615	pAR5615 carries the gene for the T7 capsid protein 10A, under the control of the *lacUV5* promoter; Amp^R	Novagen Inc., USA
pTTQ18	pUC18 derivative containing a polylinker/*lacZ*α region flanked by the *Ptac* promoter and the *rrnB* transcription terminator, $lacI^q$; Amp^R	Stark, 1987
pIB2	pTTQ18 derivative containing a *Eco*RI/*Hin*dIII *groEL* fragment subcloned from pIB1; Amp^R	This study (Figure 3.6)

Table 2.3 Table showing all the *Escherichia coli* and *Salmonella typhimurium* strains and their derivatives, used during the course of this study.

Bacterial strain	Genotype	Source/Reference
E. coli DH5α	Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169	Hanahan, 1983
DH5α (pBR325)	Amp^R Tet^R Cml^R	N. Holden
DH5α (pIB1)	Amp^R Tet^S Cml^S	This study
JM109	*endA1, recA1, gyrA96, thi, hsdR17*(r_K^-, m_K^+), *relA1, supE44*, Δ(*lac-proAB*), [F', *traD36, proAB, lacI*^{q}*ZΔM15*]	Yanisch-Perron *et* al., 1985
JM109 (pTTQ18)	Amp^R	Stark, 1987
JM109 (pIB2)	Amp^R	This study
BLT5615	pAR5615 (Amp^R); pAR5615 carries the gene for the T7 capsid protein 10A, under the control of the *lacUV5* promoter	Novagen Inc., USA
S. typhimurium SL1344	*his*; highly virulent wild-type mouse pathogen	Hoiseth and Stocker, 1981

2.1.2 Media

All media was sterilised by autoclaving unless otherwise stated.

Luria - Bertani (LB) medium and agar

LB medium consisted of bacto-tryptone (10 g; Difco Laboratories GmbH, Germany), yeast extract (5 g; Difco Laboratories GmbH, Germany) and NaCl (10 g) dissolved in 1 litre of dH_2O. LB agar was formed by adding 15 g/l bacto-agar (Difco Laboratories GmbH, Germany) prior to autoclaving.

Terrific Broth (TB)

1 litre of TB consisted of bacto-tryptone (12 g; Difco Laboratories GmbH, Germany), yeast extract (24 g; Difco Laboratories GmbH, Germany) and 4ml glycerol dissolved in deionized water.

SOC medium

1 litre of SOC broth (pH 7.0) was made from bacto-tryptone (20 g; Difco Laboratories GmbH, Germany), yeast extract (5 g; Difco Laboratories GmbH, Germany), NaCl (0.5 g), KCl (0.186 g), $MgCl_2$ (2.033 g), and glucose (3.6 g).

YT medium

YT low-salt medium consisted of bacto-tryptone (10 g; Difco Laboratories GmbH, Germany), yeast extract (5 g; Difco Laboratories GmbH, Germany) and NaCl (5 g) dissolved in 1 litre of dH_2O.

M9 minimal agar

300 ml of 15 g/l bacto-agar (Difco Laboratories GmbH, Germany) in dH_2O was autoclaved for 20 minutes and then allowed to cool down to ~ 50^0C. It was then mixed with 100 ml of 4-fold concentrated M9 salts, 8 ml 20% (w/v) glucose and 0.4 ml 1M thiamine.HCl.

LC top agar

LC top agar consisted of bacto-tryptone (10 g; Difco Laboratories GmbH, Germany), yeast extract (5 g; Difco Laboratories GmbH, Germany), NaCl (5 g) and bacto-agar (7 g; Difco Laboratories GmbH, Germany) dissolved in 1 litre of dH_2O. The pH was adjusted to pH 7.0.

M9LB medium

M9LB medium was prepared from 4-fold concentrated M9 salts (25 ml), 20% (w/v) glucose (2 ml), 1M $MgSO_4$ (0.1 ml) dissolved in 100 ml LB broth and sterilised by autoclaving. It was used to grow T7 lysates.

2.1.3 Solutions

All solutions were made up in dH_2O (unless otherwise stated) and sterilised by autoclaving at 15 pounds per square inch for 20 minutes prior to use. Heat labile components were filter-sterilised through 0.45 μm sterile filters (Pall Gelman Laboratory, USA) and then added to the initial solution after it was autoclaved.

M9 salts

4-fold concentrated M9 salts were prepared from NH_4Cl (4 g), KH_2PO_4 (12 g) and Na_2HPO_4 x $7H_2O$ (24 g) dissolved in 1 litre dH_2O.

Tris.HCl

Tris base (tris [hydroxymethyl] aminomethane) was dissolved to the desired molarity in dH_2O and the pH was adjusted to the required value by addition of concentrated HCl.

PBS

Phosphate buffered saline (PBS), pH 7.4, was prepared by dissolving 1 tablet of PBS (800 mg NaCl, 20 mg KCl, 144 mg Na_2HPO_4, 24 mg KH_2PO_4) in 100 ml dH_2O.

EDTA

EDTA (ethylenediaminetetraacetic acid, di-sodium salt) was dissolved in dH_2O to a concentration of 0.5 M and was adjusted to pH 8.0 by addition of NaOH.

TE buffer

1 litre of buffer contained 10 mM Tris.HCl (pH 8.0) and 1 mM EDTA in dH_2O.

10x TBE buffer

1 litre of buffer contained 0.9 M Tris.HCl (pH 7.5), 0.9 M boric acid and 0.02 M EDTA dissolved in dH_2O.

Ethidium bromide

A stock solution of ethidium bromide was dissolved in dH_2O at a concentration of 10 mg/ml and stored at room temperature in the dark.

6x DNA loading buffer

6x loading buffer for nucleic acid gel electrophoresis consisted of a 40% (w/v) sucrose solution, with 0.25% (w/v) bromophenol blue, in dH_2O.

GTE buffer

GTE buffer used for preparation of plasmid DNA contained 25 mM Tris.Cl (pH 8.0), 50 mM glucose and 10 mM EDTA (pH 8.0). It was stored at 4^0C.

STEP solution

Freshly prepared STEP solution, used for genomic DNA extraction, contained 0.5% (w/v) SDS in dH_2O, 50 mM Tris.HCl (pH 7.5), 0.4 M EDTA and 1 mg/ml proteinase K.

3M sodium acetate

40.824 g sodium acetate were dissolved in 80 ml dH_2O, the pH was adjusted to 5.2 with glacial acetic acid, and then the volume was adjusted to 100 ml with dH_2O.

Potassium acetate (~ pH 4.8)

To 60 ml of 5 M potassium acetate were added 11.5 ml of glacial acetic acid and 28.5 ml dH_2O. The resulting solution was 3 M with respect to potassium and 5 M with respect to acetate and was used for preparation of plasmid DNA.

IPTG

A 0.1 M stock solution was prepared by dissolving 1.2 g isopropyl β–D-thiogalactopyranoside (IPTG) in 50 ml dH_2O. The solution was filtered and then stored at 4^0C in a dark bottle.

X-Gal

A stock solution of X-Gal was prepared by dissolving 200 mg 5-bromo-4-chloro-indolyl-β-D-galactoside (X-Gal) in 10 ml dimethylformamide in a dark bottle and then stored at -20^0C.

RNase A (DNase-free) solution

A stock solution of 10 mg/ml pancreatic RNase A (from Sigma,UK) in 0.1 M Tris.HCl (pH 7.5), 0.015 M NaCl, was prepared by boiling the mixture for 15 minutes and then cooling it down to room temperature. Aliquots were stored at -20^0C.

Tris - buffered phenol

Tris - buffered phenol was obtained by mixing equal amounts of water-equilibrated phenol (from Sigma, UK) and 1 M Tris.HCl (pH 8.0), agitating vigorously and allowing the phases to separate. The process was repeated a further 2 times using 0.1 M Tris.HCl (pH 8.0). The buffered phenol was dispensed in 10 ml volumes and stored at 4^0C in light tight bottles for no longer than 2 weeks.

Tris buffered saline (TBS)

Tris buffered saline was prepared by dissolving 4.84 g Tris base and 58.48 g NaCl in 1.5 L dH_2O. The pH was adjusted to 7.5 with concentrated HCl and then the solution was made up to 2 L.

Tween - Tris buffered saline (T-TBS)

T-TBS was prepared by dissolving 0.5 ml polyoxyethylene sorbitan monolaurate (Tween 20) in 1 litre of TBS (pH 7.5).

100x Denhardt's solution

Ficoll 400 (10 g), polyvinylpyrrolidone (10 g) and bovine serum albumin (10 g) were dissolved in a total volume of 500 ml dH_2O. The solution was stored at -20^0C and used for *in situ* hybridisation experiments.

20x SSC solution

175.3 g NaCl and 88.2 g sodium citrate were dissolved in 80 ml dH_2O, the pH was adjusted to 7.0 with 10 M NaOH and then the volume was made up to 1 litre with dH_2O. The solution was used for *in situ* hybridisation experiments.

Antibiotic solutions

All antibiotic solutions were used as indicated in Table 2.4. Aqueous solutions were filter sterilised prior to use and were all stored at -20^0C. Tetracycline solution was covered with foil.

Table 2.4 Antibiotics used during this investigation.

Antibiotic	Solvent	Stock concentration	Final concentration
Ampicillin	dH_2O	10 mg/ml	100 µg/ml
Carbenicillin	dH_2O	100 mg/ml	50 µg/ml
Tetracycline	70% Ethanol	5 mg/ml	10 µg/ml
Chloramphenicol	Ethanol	34 mg/ml	25 µg/ml

2.1.4 DNA molecular weight markers

The position of DNA samples in the agarose gel was estimated in relation to DNA molecular weight markers. For sizing of a broad range of fragments (from 23,130 bp to 564 bp) and for approximate quantitation, the DNA molecular weight marker II set (from Boehringer Mannheim, Germany) was used, containing 7 bands of bacteriophage lambda (λ) DNA digested with *HindIII* endonuclease (23,130 bp, 9,416 bp, 6,557 bp, 4,361 bp, 2,322 bp, 2,027 bp and 564 bp). For sizing of a lower range of DNA fragments (from 1,500 bp to 100 bp), the 100 bp DNA ladder from New England BioLabs was used (1,500 bp, 1,200 bp, 1,000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 200 bp, 100 bp).

2.1.5 Protein molecular size markers

Molecular weight standard markers (normal and [^{14}C]-methylated) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins are indicated below in Tables 2.5, 2.6 and 2.7.

Table 2.5 Molecular weight markers (MW-SDS-70L, Sigma, UK) used for SDS-PAGE.

Protein standard	Molecular weight (Da)
Albumin (bovine)	66,000
Albumin (egg)	45,000
Glyceraldehide-3-phosphate dehydrogenase (rabbit muscle)	36,000
Carbonic anhydrase (bovine erythrocytes)	29,000
Trypsinogen (bovine pancreas)	24,000
Trypsin inhibitor (soybean)	20,100
α-Lactalbumin (bovine milk)	14,200

Table 2.6 - Molecular weight markers (SDS-6H, Sigma, UK) used for SDS-PAGE.

Protein standard	Molecular weight (Da)
Myosin	205,000
β-Galactosidase	116,000
Phosphorylase B	97,000
Bovine Serum Albumin	66,000
Ovalbumin	45,000
Carbonic Anhydrase	29,000

Table 2.7 - Rainbow [^{14}C]-methylated (4 μCi/ml) protein molecular weight markers (Amersham, UK) used for SDS-PAGE.

Protein standard	Molecular weight (Da)
Ovalbumin	46,000
Carbonic Anhydrase	30,000
Trypsin Inhibitor	21,500
Lysozyme	14,300
Aprotinin	6,500
Insulin (b) Chain	3,400
Insulin (a) Chain	2,350

2.2 METHODS

2.2.1 Manipulation of bacteria and phage

Growth of bacterial cultures

Cultures of *S. typhimurium* and *E. coli* were grown in LB, with antibiotic when appropriate, unless otherwise stated. Cultures were inoculated from a single bacterial colony and grown at 37^{0}C with shaking (300 rpm). Larger cultures were prepared by

diluting an overnight culture 100-fold in conical flasks, with an overall capacity 5-10-fold that of the culture volume, under the growth conditions stated above.

Storage of bacterial cultures

For long-term storage of bacterial cultures, a 1 ml volume of an overnight culture was mixed with 75 µl of dimethyl sulphoxide (DMSO) and stored in a sterile cryovial at -80^{0}C. For short-term storage (4-6 weeks), bacteria were stored as streaks on LB-agar plates at 4^{0}C.

In vitro packaging of T7 recombinant phage

In vitro packaging of ligation reactions was carried out with the T7 Packaging Extract, as described by the suppliers (Novagen , USA). Basically, the T7 Packaging Extract (25 µl) was thawed on ice and 5 µl ligation reaction was added. The sample was mixed gently by stirring with a pipette tip and incubated at room temperature (22^{0}C) for 2 hours. The reaction was stopped by adding 9 volumes of sterile LB and stored at 4^{0}C with 20 µl chloroform without significant loss in titre, until further use. To determine the number of recombinants generated, a plaque assay was performed.

T7 phage titration

A plating assay was used with the T7 Select System protocol to determine the efficiency of packaging reactions, to determine the number of primary recombinants and to determine the titre of amplified phage. For this purpose, the host strain BLT5615 was grown overnight at 37^{0}C on shaking in LB supplemented with 50 µg/ml Carbenicillin and then inoculated in fresh medium and incubated in the same conditions as above until OD_{600} was 0.5. IPTG (1 mM final concentration) was added and incubation continued for another 30 minutes to induce production of the 10A protein. To titre the phage, 0.25 ml culture of BLT5615 and 120 µl IPTG (0.1 M) was added to 3.0 ml of melted LC top agar at 48^{0}C. An appropriate dilution of phage (100 µl in fresh LB) was added and the contents of the tube were immediately mixed and then poured in a standard Petri dish containing hardened LB-agar. The level of dilution necessary for plating depended on the source of the sample, for packaging reactions the dilution range was 10^{-4} – 10^{-7} and for an amplified lysate the

dilution range was 10^{-7} – 10^{-10}. Clear areas (plaques) were observed in the lawn of host bacteria following overnight incubation at room temperature, which corresponded to individual phage infection events.

Amplification of the T7 bacteriophage

After *in vitro* packaging of T7 phage and plaque assay to determine the T7 titre, a plate lysate amplification experiment was carried out. 1 ml from an overnight starter culture of BLT5615 in LB (Carbenicillin supplemented) was inoculated into 50 ml LB in a conical flask (250 ml) and incubated at 37^0C with shaking, until OD_{600} reached 0.5. IPTG (to 1 mM final concentration) was added 30 minutes prior to infection of cells at a ratio of 10^5 phage particles per 1 ml cells. This mixture was plated with IPTG and molten LC top agar at 48^0C onto a LB-agar plate containing Carbenicillin, and plates were incubated overnight at room temperature. The phage was harvested by covering each plate with Phage Extraction Buffer (20 mM Tris.HCl [pH 8.0] containing 100 mM NaCl and 6 mM $MgSO_4$), and incubating the plates overnight at 4^0C. The lysate was gently mixed with 0.5 ml chloroform, then clarified by centrifugation at 3,000 g for 5 minutes and the supernatant was collected, titred by plaque assay and stored at 4^0C for several months without a loss of titre.

Growth and storage of T7 lysates

At an OD_{600} of approximately 0.5, IPTG (1 mM final concentration) was added to a culture of BLT5615 growing logarithmically in M9LB. After 30 minutes, phage were added and the culture was incubated until lysis. NaCl (0.5 M final concentration) was added and the culture was centrifuged at approximately 12,000 g in a Beckman J2-21 (JA-20 rotor). The clarified lysates could be stored stably for months to years at 4^0C. For long-term storage, samples of the clarified lysate were combined with 0.1 volume of sterile 80% glycerol and then frozen at -80^0C. These stocks can be frozen and thawed with little loss of titre.

Purification of T7 from lysates

T7 recombinant phage was purified from different volumes of clarified lysates by precipitation with polyethylene glycol (PEG 8,000 from Sigma, UK) followed by banding in a CsCl step gradient. In each case, phages were initially extracted from the PEG pellet in 10 mM Tris.HCl (pH 8.0) containing 1M NaCl and 1 mM EDTA, and the concentrated phage solution was layered on top of a 4-step CsCl gradient. The CsCl layers were made by mixing a stock solution of 62.5% (w/v) CsCl with TE buffer (10 mM Tris.Cl, pH 8.0, containing 1 mM EDTA) in the following ratios in SW41 centrifuge tubes: 2 ml of 1:2 (CsCl:TE), 2 ml 1:1 (CsCl:TE), 2 ml 2:1 (CsCl:TE) and 1 ml 1:0 (CsCl:TE). Successively denser solutions were gently underlayered in the SW41 tube using a pipette. After centrifugation, the tubes contained a thick layer of debris and empty phage heads on top of the 1:2 layer, a sharp, turbid band of phage particles above the 2:1 layer, and a lower turbid band (carbohydrate fraction with no phage particles) above the 1:0 layer. The tube was punctured below the desired band which was collected with a syringe needle. Purified phage particles were stored in CsCl solution at 4^0C after performing a plaque assay.

- *Purification of T7 from 35 ml-lysates:* 0.15 ml of BLT5615 overnight culture grown in LB (Carbenicillin supplemented) was added to 35 ml M9LB medium (Carbenicillin supplemented), incubated again at 37^0C, with shaking, until the OD_{600} reached 0.5 and then IPTG (1 mM final concentration) was added 30 minutes before phage infection. An agar plug containing a single T7 plaque was added and shaking was continued at 37^0C until lysis (clearing of the culture) was observed. 3.0 ml of 5M NaCl solution was added and the culture was centrifuged at approximately 6,000 g in a Beckman J2-21 (JA-20 rotor) for 10 minutes at 4^0C. The remaining supernatant was poured into a fresh tube and 1/6 volume of sterile 50% (w/v) PEG 8,000 in dH_2O was added, followed by thorough mixing by vortexing. The lysate-PEG mixture was allowed to stand overnight at 4^0C, to precipitate the phage particles, and then was centrifuged at 6,000 g for 10 minutes. 1.2 ml of 10 mM Tris.Cl (pH 8.0) containing 1M NaCl and 1mM EDTA, was added to the drained pellet. This was vortexed and then centrifuged at 12,000 g for 10 minutes. The supernatant was layered on the CsCl step

gradient in an SW41 tube and then ultracentrifuged at 35,000 rpm for 60 minutes at 20^0C in a Beckman ultracentrifuge (SW41 swinging bucket rotor). The phage band was removed as described.

- *Purification of T7 from 2 litre*-lysates: Large amounts of T7 phage were purified from 2 litres of lysate as 4 x 500 ml lysates in 2-litre flasks. A fresh overnight BLT5615 culture was diluted 1:200 in 500 ml of fresh M9LB (Carbenicillin supplemented) and grown at 37^0C until OD_{600} reached 0.5. After 30 minutes incubation with IPTG (1 mM final concentration), the culture was infected with 50 µl of high titre phage lysate and shaken at 37^0C until lysis. 100 µl DNase I (1 mg/ml) was then added to each flask and incubation was continued for 15 minutes. 12.5 g solid NaCl was then dissolved in each flask and the suspension was centrifuged at 4^0C for 10 minutes at 9,800 g in a Beckman J2-21 (JA-14 rotor). 200 g PEG 8,000 was dissolved in the combined supernatants, the suspension was left overnight at 4^0C and then centrifuged at 4^0C for 10 minutes at 9,800 g. The pellets were then resuspended in 40 ml of 10% PEG (10 g PEG 8,000 in 100 ml of 10 mM Tris.HCl [pH 8.0] containing 1 mM EDTA) and centrifuged at 4^0C for 10 minutes at 5,900 g in a Beckman J2-21 (JA-20 rotor). Pellets were resuspended in 5 ml of 10 mM Tris.HCl (pH 8.0) containing 1 mM EDTA and 1 M NaCl, mixed by vortexing, re-centrifuged as above and the supernatants layered on the CsCl step gradient in an SW41 tube, then ultracentrifuged and the phage band collected.

In situ hybridisation of bacteriophage T7 plaques

Screening of recombinant T7 bacteriophage plaques was promoted by hybridisation with a ^{32}P-labelled DNA probe (Benton and Davis, 1977). Bacteriophages were plated at an appropriate density (approximately 500 plaques per 90 mm plate) and an imprint of the pattern of plaques was obtained by gently layering a nitro-cellulose membrane onto the surface of the LC top agar. Bacteriophage particles and DNA were transferred to the membrane by capillary action. After denaturation for 5 minutes with alkali (0.5 N NaOH, 1.5 M NaCl) and neutralisation (1.5 M NaCl, 0.5 M Tris.HCl, pH 7.4) for another 5 minutes, membranes were rinsed in 2x SSC

solution. The membranes were then air-dried for 60 minutes at room temperature, and bacteriophage particles and DNA were irreversibly bonded to the membranes by baking for 1½ hours at 80^{0}C in a vacuum oven. The membranes were incubated in 20 ml of a pre-hybridisation buffer composed of 6x SSC, 0.5% (w/v) SDS, 50x Denhardt's solution and 100 µg/ml denatured salmon sperm in a hybridisation roller in the oven at 68^{0}C. After 1½ hours, the pre-hybridisation mixture was replaced with an equal volume of fresh buffer, along with 25 µl of probe, which was denatured by boiling for 10 minutes. Hybridisation was allowed to proceed overnight at 68^{0}C, after which the membranes were washed two times with Wash I solution (2x SSC, 0.1% SDS [w/v] in dH_2O) at 68^{0}C and for two times with Wash II solution (1x SSC, 0.1% SDS [w/v] in dH_2O) at 68^{0}C, each time for approximately 1½ hours, with rotation. The membranes were then wrapped in plastic foil and exposed overnight to an autoradiographic film at -80^{0}C. Hybridising plaques, identified by aligning the films with the original agar plates, were picked for further analysis.

Preparation of T7 DNA

T7 DNA was prepared from the CsCl-banded phage. 10 volumes of 10 mM EDTA (pH 8.0) were added to 1 volume phage. The mixture was heated at 65^{0}C for 5 minutes and then cooled down to room temperature. After addition of 2.5 volumes of absolute ethanol and incubation at –20^{0}C for 20 minutes, the sample was centrifuged for 5 minutes at 10,000 g. The pellet was rinsed twice with 500 µl 70% ethanol, dried and then dissolved in TE buffer for 1-2 hours at 37^{0}C.

2.2.2 Nucleic acid manipulation and detection methods

Agarose gel electrophoresis

For both preparative and analytical purposes DNA was resolved by electrophoresis in 0.8% to 2% (w/v) agarose gels, as appropriate, containing 0.5 µg/ml ethidium bromide. Samples were loaded with 1/6th volume of 6x loading buffer (40% [w/v] sucrose solution in dH_2O, with 0.25% [w/v] bromophenol blue) and electrophoresed at 100V in a 110 mm x 150 mm horizontal gel unless otherwise indicated, until

suitable resolution had been obtained. DNA was visualised under UV light (λ 313 nm) and photographed using a Mitsubishi video copy processor.

Phenol/chloroform extraction and ethanol precipitation of DNA

Phenol/chloroform extraction and ethanol precipitation of DNA was performed to remove cell debris and proteins associated with DNA, as standardly described by Sambrook and co-workers (1989).

Preparation of plasmid DNA

Plasmid DNA was prepared by alkaline lysis (Birnboim and Doly, 1979; Sambrook *et* al., 1989). For small-scale preparations, DNA was extracted from 1.5 ml of overnight culture. The final DNA pellet was washed with 1 ml 70% ethanol, dried under vacuum and then gently resuspended in 50 µl TE (pH 8.0) containing DNase-free pancreatic RNase (20 µg/ml) and stored at -20^{0}C until use.

To obtain large amounts of very pure plasmid DNA, the protocols of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) were employed using 500 ml culture and with the following variations. After addition of NaOH (0.2 N) and SDS (1% [w/v] in dH_2O) ice-cold potassium acetate (~ pH 4.8) solution was used for neutralisation. After centrifugation at 6,000 g for 10 minutes at 4^{0}C to pellet the cell debris, the supernatant was filtered through a non-absorbent cotton wool plug and the filtrate was collected in a clean centrifuge pot where 48 ml isopropanol were added to precipitate the DNA. The plasmid DNA was harvested by centrifugation at 6,000 g for 10 minutes at 4^{0}C, the pellet was rinsed with 70% (v/v) ethanol and was air dried by inversion of the centrifuge pot, to remove excess liquid. The dried pellet was dissolved in 9 ml TE buffer (pH 7.5) and then 9.2 g of CsCl were added, followed by addition of 0.5 ml of ethidium bromide solution (10 mg/ml). At this stage, the tube containing the DNA and ethidium bromide mixture was wrapped in foil to avoid contact with light. The sample was centrifuged for 10 minutes at 4,000 g in a MSE Centaur I centrifuge to remove debris. The cleared supernatant was then spun in Sorvall TV865 quickseal ultracentrifuge tubes at 200,000 g for 20 hours at room temperature, in a Sorvall OTD55B ultracentrifuge. Using a sterile needle and

syringe, the lower band containing plasmid DNA was removed after visualisation under UV light. Extraction of ethidium bromide from the sample was performed several times by mixing with TE-saturated butan-1-ol and discarding the upper (butanol/ethidium bromide) layer. The aqueous phase was then dialysed overnight against TE (pH 8.0) to remove CsCl. The DNA was ethanol precipitated and the final pellet was dried under vacuum and resuspended in TE (pH 8.0). As an alternative to this protocol, the Qiagen Plasmid Maxi Kit (Qiagen GmbH, Germany) was used, following the manufacturers recommendations. This was based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin, elution in a high-salt buffer and isopropanol precipitation.

Preparation of dialysis tubing:

Dialysis membrane (Medicell International Ltd.) was prepared by boiling for 10 minutes in a 2% (w/v) sodium bicarbonate solution in dH_2O containing 0.05% (w/v) EDTA. This solution was then discarded and the membrane then boiled twice in dH_2O. After this final step, the membrane was transferred to a solution containing 20% (v/v) ethanol in dH_2O and stored at 4^0C until further use. Prior to dialysis, the membrane was rinsed with sterile dH_2O to remove the ethanol.

Preparation of genomic DNA from *S. typhimurium* SL1344

1.5 ml of overnight culture was transferred to a clean tube and cells were harvested by centrifugation at 20,000 g for 10 minutes. Cells were resuspended in 0.5 ml of solution containing 50 mM Tris.HCl (pH 8.0) and 50 mM EDTA and frozen at -20^0C for 30 minutes. 0.05 ml fresh 10 mg/ml lysozyme solution in 0.25 M Tris.HCl (pH 8.0) was added to the frozen cells which were thawed quickly by hand. When just thawed, this suspension was left on ice for 45 minutes, then 0.1 ml STEP solution (0.5% [w/v] SDS solution in dH_2O containing 50 mM Tris.HCl (pH 7.5), 0.4 M EDTA and 1 mg/ml proteinase K) was added, with incubation at 50^0C for 60 minutes with occasional mixing. This suspension was then split in half in two micro-centrifuge tubes and to each tube 2 volumes of Tris-buffered phenol were added. The mixture was gently combined by inverting the tubes until an emulsion was formed and then centrifuged at 4^0C in a micro-centrifuge at 10,000 g for 15 minutes.

The upper aqueous layer which contains the DNA was then transferred into a new tube using a blunt-end pipette tip. The procedure was repeated with phenol:chloroform:isoamylalcohol (25 : 24 : 1) until a clear interface was observed. The aqueous layer was then transferred into a new tube and the DNA was precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% cold ethanol. Precipitated DNA was then spooled out and transferred to a clean tube containing 0.5 ml of 200 μg/ml RNase A (DNase free) in 50 mM Tris.HCl (pH 7.5) containing 1 mM EDTA. The tube was rotated gently overnight at 4^0C. An equal volume of chloroform was added, the tube was inverted for 5 minutes to emulsify and then centrifuged in a micro-centrifuge at 10,000 g for 15 minutes at 4^0C. The aqueous top layer was then transferred to a clean tube and the DNA was precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% cold ethanol. The tube was gently inverted several times until a stringy white precipitate of DNA appeared. The DNA was spooled out and added to a solution of 50 mM Tris.HCl (pH 7.5) containing 1mM EDTA.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) (Saiki *et* al., 1988) was employed in order to amplify DNA fragments for a variety of applications; the reaction was performed in a Techne "Gene E" thermal cycler dry-block. Primers specific for the region of DNA of interest were designed as required (Table 2.1). PCR reaction products were then analysed by agarose gel electrophoresis.

The *groEL* fragment was amplified over 30 cycles consisting of 3 steps: denaturation at 94^0C for 1 minute, annealing at 60^0C for 1 minute and elongation at 72^0C for 2 minutes. A final extension cycle was performed for 10 minutes at 72^0C. Reactions were carried out in 100 μl volumes containing template DNA (10 ng), 2.5 μl of each primer (at a concentration of 3.2 pmol/μl each), 8 μl of dNTP mix (containing 2.5 mM of each dNTP), 2 units of Taq DNA polymerase (Boehringer Mannheim), 10 μl of a 10x PCR reaction buffer (Boehringer Mannheim) and dH_2O. In order to avoid evaporation of sample through the PCR process, sterile mineral oil (100 μl) was added to the surface of the PCR mixture.

For T7 DNA, a portion of the LC top agar, scraped from an individual plaque, was dispersed into a tube containing 100 µl of 10 mM EDTA (pH 8.0), mixed by vortexing and then heated at 65^0C for 10 minutes. Samples were centrifuged at 14,000 g for 3 minutes to clarify and the phage lysate was then combined with PCR reaction buffer and components in a 50 µl total volume. PCR amplification was performed by starting with a single denaturation step at 94^0C for 4 minutes, followed by 35 cycles comprising: denaturation at 94^0C for 1 minute, annealing at 50^0C for 1 minute and elongation at 72^0C for 2 minutes. The final extension consisted of a single cycle at 72^0C for 6 minutes.

Transformation of *E. coli* by electroporation

An overnight culture was diluted 1/100 in 500 ml fresh LB broth and grown at 37^0C with shaking until the OD_{600} had reached 0.5. The flask was chilled on ice for 1 hour and then cells were centrifuged at 4,000 g for 10 minutes at 4^0C in a Beckman JA-21 (JA-14 rotor). The supernatant was discarded, the cells were resuspended in an equivalent volume of ice-cold sterile dH_2O and this process was repeated 4 times. The cells were centrifuged at 4^0C for 10 minutes at 4,000 g. After the final spin, the cells were resuspended in 0.5 ml dH_2O and used immediately for electroporation as 200 µl aliquots in pre-chilled electroporation cuvettes. Cells kept for further transformations were stored as 200 µl aliquots at -80^0C in 10% glycerol for no more than 3 weeks.

Between 5 pg and 10 ng DNA in less than 10 µl volume, was added to competent cells in electroporation cuvettes and left on ice for 5 minutes. Electroporation was performed using the BIORAD "Gene Pulser" apparatus, with the voltage set at 2.5 kV and the pulse controller set to 200 ohms. After the pulse, 800 µl of SOC medium was added to the cuvettes and the whole contents were transferred to a fresh microcentrifuge tube. The cells were then incubated at 37^0C or 30^0C for 1 hour, prior to plating on selective LB-agar.

$CaCl_2$ method for transformation of *E. coli*

This protocol was used to prepare JM109 competent cells. The strain was grown on M9 minimal plates prior to preparing competent cells and transformation, then a single colony was inoculated in 5 ml YT medium (low salt concentration) and incubated overnight at 37^0C with shaking. The overnight culture was diluted 1/100 in 50 ml fresh warm YT medium and grown in the same conditions (with good aeration) until OD_{600} was approximately 0.5. The cells were pelleted by centrifugation at 4^0C for 10 minutes at 7,000 g in a Beckman JA-21 (JA-20 rotor), the supernatant discarded and then the cells resuspended in approximately 25 ml of pre-chilled 10 mM Tris.HCl (pH 8.0) containing 50 mM $CaCl_2$. After 30 minutes incubation on ice, cells were centrifuged at 4^0C for 10 minutes at 7,000 g, resuspended in 2.5 ml pre-chilled $CaCl_2$ / Tris mixture and incubated on ice between 30 minutes and 2 hours prior to transformation.

A 100µl aliquot of JM109 competent cells prepared as above was mixed gently by swirling with ~ 10 ng DNA (approximately 5 µl of the ligation reaction) and then incubated on ice for 30-40 minutes. The tube was heated at 42^0C in a water bath for 50 seconds and then returned to ice for at least 2 minutes. 4 volumes of fresh LB was added to the cells and then incubated at 37^0C for 60 minutes. This suspension was then spread in 200µl aliquots onto LB plates (with 50 µg/ml Carbenicillin) containing X-Gal and IPTG and then incubated overnight at 37^0C.

DNA sequencing

DNA sequencing was performed by an automated and non-isotopic method first described by Ansorge and colleagues (1986), using fluorescent dye terminators from the Big Dye Terminator Kit (Applied Biosystems, USA).

PCR products and plasmid dsDNAs were used as templates in the Big Dye Terminator cycle sequencing reactions. For automated fluorescent DNA sequencing purposes, plasmid DNA was obtained with Wizard Plus SV Minipreps DNA Purification System kit, as described by the manufacturers (Promega, USA). After elution of plasmid DNA from the spin column, an ethanol precipitation step was

performed to concentrate the DNA. The pellet was resuspended in dH_2O and the DNA concentration determined by agarose gel/ethidium bromide quantitation. PCR products were purified prior to cycle sequencing, by silica-based gel filtration using the Qiaquick PCR Purification Kit (Qiagen GmbH, Germany). 30 to 90 ng PCR products and between 200 to 500 ng plasmid DNA were used as templates in cycle sequencing. DNA sequencing was carried out in 10 µl volume containing template DNA, 5 pmol primer for sequencing and 4 µl of terminator mix (A-dye terminator, T-dye terminator, C-dye terminator, G-dye terminators, dATP, dTTP, dCTP, dGTP, Tris.HCl [pH 9.0], $MgCl_2$, thermostable pyrophosphatase and AmpliTaq DNA polymerase), supplied as part of the kit. Reactions were cycle sequenced in a Techne "Gene E" thermal cycler dry-block over 25 cycles, each cycle including 30 seconds at 96^0C, 20 seconds at 50^0C and 4 minutes at 60^0C. Dye terminators and excess nucleotides were removed using Centriflex Gel Filtration Columns (Edge BioSystems, Inc.) prior to processing on the automated DNA sequencer. Raw data were analysed using GeneJockey (Biosoft) software.

Radioisotopic labelling of DNA

Plasmid DNA was labelled by incorporation of labelled deoxynucleotide triphosphates using the Nick Translation System (Promega Inc, USA). Approximately 1 µg plasmid DNA was mixed with 5 µl [α-^{32}P]-dCTP (10 µCi/µl and 400 Ci/mmole), 5 µl of a DNA polymerase I/DNase I mix, 10 µl nucleotide mix (300 µM dATP, 300 µM dGTP, 300 µM dTTP) and made up to 50 µl in translation buffer (50 mM Tris.HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT). The DNA polymerase/DNase I mix contained: DNA polymerase I (1 U/µl) and DNase I (0.2 ng/µl) in a solution containing 50% glycerol, 50 mM Tris.HCl (pH 7.2), 10 mM $MgSO_4$, 0.1 mM DTT and 0.5 mg/ml nuclease-free BSA. The reaction was allowed to proceed at 16^0C for 60 minutes and was then stopped by the addition of 5 µl 0.25 M EDTA (pH 8.0). The reaction volume was then made up to 200 µl and was separated through 1 ml of a TE-equilibrated pre-spun Sephadex G 50 column by centrifugation at 2,000 g for 4 minutes. Prior to hybridisation the probe was denatured by boiling for 5 minutes, cooled immediately on ice and added to the hybridisation mix.

2.2.3. Protein detection procedures

Estimation of protein concentration

The concentration of a protein in solution was estimated using the method of Bradford (1976), as adapted by the reagent manufacturers (BIORAD). Standard bovine serum albumin (BSA) solutions of 0.2 mg/ml, 0.5 mg/ml, 0.8 mg/ml, 1.0 mg/ml and 1.25 mg/ml were prepared in the same buffer solution as used to dilute the protein sample and 2.5 ml diluted Bradford reagent was added to either 50 µl from each standard or 50 µl buffer (used as the blank sample), with gentle mixing. Each mixture was incubated at room temperature for 10 minutes and the optical density was read at 595 nm (OD_{595}). For each BSA standard, triplicate measurements of the OD_{595} were obtained and the average reading plotted against concentration to give a standard curve (data not shown). The protein concentration of the solution of interest was determined from the optical density, by using the OD reading in conjunction with the standard curve.

SDS-polyacrylamide gel electrophoresis of proteins

SDS-polyacrylamide gel electrophoresis of proteins (SDS-PAGE) was performed essentially as described by Laemmli (1970). Proteins were resolved on either a 16 x 20 cm or a 7 x 8 cm SDS-polyacrylamide gel, using a PROTEAN II xi Slab Gel or a MINI-PROTEAN II Dual Slab Gel apparatus respectively (BIORAD). The composition of the resolving gel was dependent upon the protein to be investigated and either a 12.5%, 10% or 7.5% (w/v) acrylamide gel was used, with a stacking gel at 4% (w/v) polyacrylamide concentration. The composition of the stacking gel was: 4% (w/v) polyacrylamide, 0.1 % (w/v) SDS (sodium dodecyl sulphate), 0.05 % (w/v) APS (ammonium persulphate) and 0.05% (w/v) TEMED (N, N, N'-tetramethylethylethylenediamine) in 0.125 M Tris.HCl buffer (pH 6.8). The resolving gels contained either 12.5%, 10% or 7.5% (w/v) polyacrylamide and 0.1 % (w/v) SDS, 0.05 % (w/v) APS, 0.05% (w/v) TEMED in 0.125 M Tris.HCl buffer (pH 8.8).

Samples to be analysed were mixed with an equal volume of 2x Laemmli solubilisation buffer (LSB) and boiled for 5 minutes prior to loading. 2x LSB contained 100 mM Tris.HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% (w/v) SDS (electrophoresis grade), 0.2% (w/v) bromophenol blue and 20% (w/v) glycerol. Boiled samples were loaded (10-30 µl, depending upon the gel used) into individual wells and the proteins were electrophoresed in running buffer (0.025 M Tris-base [pH 8.3] containing 0.192 M Glycine, 0.1% [w/v] SDS). Molecular size markers were used for sizing the polypeptides seen on the gel. Small gels (MINIPROTEAN II) were run at room temperature for 2.5 hours at 15 mA/gel and 250 V and big gels (PROTEAN II) were run at 4^0C for 18 hours at 30 mA/gel and 400 V.

Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue or Silver stain

Once the polypeptides had been separated by electrophoresis, the gels were immersed in a fixing/staining solution (0.1% [w/v] Coomassie Brilliant Blue R250 (Sigma, UK), 45% [v/v] methanol, 10% [v/v] glacial acetic acid in dH_2O) and left to stain on a shaking platform at room temperature for approximately 4 hours. Excess stain was then removed by soaking the gel in a destaining solution (45% [v/v] methanol, 10% [v/v] glacial acetic acid in dH_2O) for 4-8 hours at room temperature until polypeptide bands could be seen clearly. After destaining, gels were soaked overnight in a pre-dry solution (35% [v/v] ethanol, 0.15% [v/v] glycerol in dH_2O) and then dried at room temperature using a drying kit (Promega Inc., USA).

Silver staining of proteins resolved by SDS-PAGE was done according to the method described by Blum and collaborators (1987). To prevent diffusion of separated proteins, gels were soaked overnight in a fixing solution (50% [v/v] methanol, 12% [v/v] acetic acid and 0.5 ml 37% [v/v] formaldehyde in dH_2O) and then washed 3 times, each time for 20 minutes, in a solution of 50% (v/v) ethanol in dH_2O. Gels were then pre-treated for 1 minute in a freshly prepared solution containing 0.2 mg/ml Na thiosulphate ($Na_2S_2O_3.5H_2O$) in dH_2O, in order to get an image enhancement of fixed gels and to prevent background staining from formation of soluble silver complexes. After rinsing the gels 3 times with dH_2O, each time for 20 seconds, they were impregnated for 20 minutes with a freshly prepared solution

of $AgNO_3$ (2 mg/ml), 1 litre of solution containing 0.75 ml 37% (v/v) formaldehyde. For image development, pre-treated gels were rinsed as above and then soaked for 2-5 minutes in fresh developing solution (60 g Na_2CO_3, 4 mg $Na_2S_2O_3.5H_2O$ and 0.5 ml 37% [v/v] formaldehyde in dH_2O). When the desired contrast was achieved, the reaction was stopped by soaking the gels for 10 minutes in an aqueous solution containing 50% (v/v) methanol and 12% (v/v) acetic acid. Gels were stored at 4^0C for no longer than 2 weeks in 50% (v/v) methanol in dH_2O, prior to drying.

[^{35}S]-methionine labelling of proteins from the T7 bacteriophage

T7 proteins were labelled essentially as described previously by Studier (1972). An overnight culture of BLT5615 in LB (supplemented with Carbenicillin) was diluted 1:50 in fresh LB/Carbenicillin medium and grown with shaking at 37^0C to an absorbance of 0.5 at 600 nm (OD_{600}). This corresponded to approximately 2 x 10^8 cells/ml. 1 mM IPTG was added 30 minutes prior to T7 infection to induce plasmid production of the 10A protein. The culture was dispensed in a Petri dish, was irradiated for 6 minutes with U. V. light from a germicidal lamp located approximately 60 cm above the sample, as described by Hosoda and Levinthal (1968), and was then incubated for 15 minutes at 37^0C on shaking before infection. The cell suspension was then infected with CsCl-purified phage to give approximately 10 phage particles per cell and was incubated at 37^0C. Infection was allowed for 8 minutes, and then every 2 minutes, 1 ml-samples of culture were taken and pulsed for 2 minutes with ^{35}S-methionine at a final concentration of 15 μCi/ml (1175 Ci/mmol). The cells were collected by centrifugation for 2 minutes at 12,000 g and then resuspended in 1 ml fresh unlabelled LB in order to eliminate further incorporation of ^{35}S into proteins, followed by incubation for 4 minutes at 37^0C. The cells were harvested by centrifugation for 5 minutes at 12,000 g, lysed by boiling for 5 minutes in Laemmli solubilisation buffer (2 x LSB) and then the proteins subjected to SDS-PAGE, followed by autoradiography.

2.2.4 Immunodetection of polypeptides

Immunological detection of proteins (Western blotting) was carried out following SDS-PAGE and transfer to a solid support (*e.g.* nitro-cellulose membrane) which then could be stained (Towbin *et* al., 1979). The membrane was subsequently exposed to unlabeled antibodies specific for the target protein and finally the bound antibody was detected by an anti-immunoglobulin coupled to horseradish peroxidase (HRP). Visualisation of the protein band was performed by chemiluminescent detection when horseradish peroxidase (HRP) catalyses the oxidation of luminol in the presence of hydrogen peroxide (H_2O_2). Immediately following the oxidation, the luminol is in an excited state, and then decays to the ground state via a light emitting pathway (Thorpe and Kricka, 1986). The light signal was captured on X-ray film.

Transfer of polypeptides to nitro-cellulose membranes by electrophoresis

Western blotting was carried out by direct electrophoretic transfer of proteins from the gel to a nitro-cellulose membrane, using a "TRANS-BLOT CELL" or a "TRANS-BLOT SD SEMI-DRY CELL", as described by the manufacturers (BIORAD). Nitro-cellulose membrane (Schleicher and Schuell) was used as solid support and transfer buffer (48 mM Tris-base, 39 mM Glycine, 20% (v/v) methanol, 0.0375% (w/v) SDS in dH_2O) was used for electrotransfer. With the "TRANS-BLOT CELL", polypeptides were then transferred from the gel to the membrane by electrophoresis at 4^0C for 20 hours at 20 V (250 mA). When the "TRANS-BLOT SEMI-DRY CELL" was used, transfer of the polypeptides was carried out at 4^0C and was completed in 30 minutes to 1 hour (depending on the size of the gel) at 15 V (200 mA). After blotting, the membrane was used immediately for immunodetection experiments.

Immunodetection of polypeptides

The nitro-cellulose membrane was stained with the reversible dye Ponceau S (0.5 g in 100 ml 1% [v/v] acetic acid in dH_2O) incubating the membrane for 5-10 minutes with gentle agitation. When the protein bands were visible, the positions of molecular size marker proteins were marked in pencil. After washing in several

changes of dH_2O at room temperature, the blotted membrane carrying the proteins was shaken for 1 hour at room temperature in a 5% (w/v) non-fat dry milk solution (Sainsbury's own brand) in TBS (Tris buffer saline, pH 7.5). The membrane was then rinsed 3 times (each time for 10 minutes) in fresh changes of T-TBS (Tween-TBS) and primary antibody was added with shaking at room temperature for 1 hour in a 1% (w/v) solution of non-fat dry milk in T-TBS. The primary antibody was used either as a dilution of anti-GroEL antibody (IgG fraction) raised in rabbit (from Sigma, UK), or of mouse antisera from *Salmonella typhimurium (aroA⁻)*-immunised mice. After this incubation with the first antibody, the membrane was washed 3 times (each time for 10 minutes) in fresh changes of T-TBS and then the second antibody was added and incubation was continued for 1 hour at room temperature in a 1% (w/v) non-fat dry milk solution in T-TBS. The second antibody was represented by a 1:10,000 dilution of (sheep/donkey) anti-IgG (mouse, rabbit) conjugated to horseradish peroxidase (from SAPU, Scotland, UK). After incubation, unbound antibodies were removed by washing 3 times in T-TBS, followed by an additional washing step in TBS for another 5 minutes. Bound antibodies were detected by the addition of a developing mix (1 volume solution B, 100 volumes solution A) and allowing the reaction to proceed for 1 minute at room temperature. Solution A was prepared by dissolving 50 mg of 5-amino-2,3-dihydro-1,4-phtalazinedione sodium salt (sodium luminol, Sigma, UK) in 200 ml 0.1 M Tris.HCl (pH 8.6) containing 62 µl of 30% (v/v) H_2O_2 and was stored in a dark bottle at 4^0C. Solution B was made by dissolving 11 mg of parahydroxycoumaric acid (Sigma, UK) in 10 ml dimethyl sulphoxide (DMSO, Sigma, UK) and was stored at 4^0C as 0.5 ml aliquots. To visualise the immunoblots, membranes were wrapped in Saran Wrap and exposed to X-ray films (AGFA Curix RP1, 100 NIF) for 30 seconds to 2 minutes. X-ray films were then developed in an X-ray film auto-processor and scanned.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA (enzyme-linked immunosorbent assay) was performed essentially as described previously (Engvall and Perlman, 1971). Ninety-six well microtitre plates

(Immulon 2, Dynex) were coated with the purified protein at a concentration of 1μg/ml (100 ng antigen per well in 100 μl coating buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5]), unless otherwise specified, and were incubated overnight at 4^0C. The plates were then washed 3 times with washing buffer (0.05% [v/v] Tween 20 in PBS) to remove excess antigen, and unoccupied protein binding sites were blocked for 5 hours at room temperature with 200 μl per well blocking buffer (1% [w/v] skimmed milk powder in washing buffer). After 3 washes, doubling dilutions of serum samples, made up in blocking buffer, were added to duplicate antigen coated wells (100 μl per well) and incubated overnight at 4^0C. After 3 washes, the wells were incubated for 30 minutes at 37^0C with 100 μl per well of horseradish peroxidase-conjugated anti-IgG antibody (SAPU, Scotland, UK) at a dilution of 1:500 in blocking buffer. The plates were washed further 3 times with washing buffer before incubating for 15 minutes at room temperature with 100 μl per well of substrate buffer. The substrate buffer (10 ml) contained 2.4 ml of 0.1 M citric acid, 2.56 ml of 0.2 M Na_2HPO_4, 4 μl of 30% (v/v) H_2O_2, 5 ml dH_2O and 4 mg *O*-phenylenediamine (OPD, from Sigma). The reaction was stopped with 25 μl of 2M H_2SO_4 and the absorbance was read at 492 nm using a Titertek Multiskan Plus plate reader and the software ELISA LITE v. 3.01.03 (1986-1991 Meddata Inc.).

2.2.6 Murine model studies

The T7 recombinant bacteriophage and purified GroEL from *Salmonella typhimurium* were used as immunogens to produce polyclonal antisera in female BALB/c mice (approximately 6 weeks of age).

Obtaining T7 bacteriophage-specific serum from mice

20 mice (five per group) were injected by the i.v. route with 100 μl of PBS containing 10^{11} pfu/ml CsCl-purified T7 recombinant phage (10^{10} pfu/mouse) on day 1 and day 14. Mice were bled prior to infection (day 0), on day 10 after the first immunisation, and on days 6 and 12 after the second immunisation (days 20 and 26, respectively). Approximately 100 μl of blood was obtained per mouse. The blood was allowed to clot at room temperature for 5-6 hours and the serum was then

removed from the clot and any remaining insoluble material by spinning the samples at 1,800 g in a MSE Centaur bench-top centrifuge for 15 minutes. Serum was aliquoted, stored at -80^0C and then serum samples from individual mice were examined.

Preparation of anti-GroEL (from *S. typhimurium*) antisera obtained from mice

Purified GroEL from *Salmonella typhimurium* prepared by C. Aspinwall as previously described (Taylor, 1997), was diluted to 500 μg/ml in sterile PBS. 3.2 ml of this solution was mixed with an equal volume of adjuvant (Imject Alum, Pierce, USA) added dropwise with stirring for 30 minutes at room temperature. A group of 6 mice was injected with 200 μl per mouse of the GroEL-Alum mixture (50 μg protein per mouse) with each dose being distributed equally between two separate subcutaneous sites. 100 μl injection of mouse recombinant interleukin-12 (IL-12, R&D Systems, UK) was also given intraperitoneally (2.5 μg/ml IL-12). The other groups of mice were injected subcutaneously either with 50 μg GroEL in Alum per mouse (as before), or the control groups of mice were injected solely with 100 μl PBS or 100 μl IL-12 (2.5 μg/ml) at the appropriate site. After an interval of 14 days, each group of mice were given a repeat immunising dose and samples of blood were collected under anaesthesia on days 6 and 12 after the second immunisation. The blood from each subgroup of mice was pooled and the serum was obtained and stored as described above.

CHAPTER 3

Cloning and characterisation of *S. typhimurium* GroEL

3.1 INTRODUCTION

3.1.1 *S. typhimurium* GroEL – an immunogen used in vaccine design

When bacteria are subjected to environmental stress they respond by increasing the level of various proteins, which in turn help them deal with the detrimental effects of exposure to the stress (Mahan *et* al., 1996). For example, when *S. typhimurium* enters the macrophage, the bacterium responds by inducing around 30-40 proteins and repressing around 100 (Buchmeier and Heffron, 1990; Abshire and Neidhardt, 1993). Stress proteins represent some of the most conserved and abundant proteins in nature, and appear to have a variety of important roles in immunity. The elevated synthesis of stress proteins in host phagocytes also increases their availability for antigen recognition by the host immune system. It has been suggested that such stress proteins may be dominant antigens for recognition by both cell-mediated and humoral immune responses (Kagaya *et* al., 1992).

The GroEL protein is an abundant protein of the bacterial cell. In *E.coli*, GroEL constitutes approximately 1% of the total protein content in unstressed cells, but this can be elevated to approximately 10% under stress conditions (Hemmingsen *et* al., 1988). It has been estimated that at least half of the soluble proteins of *E. coli* can form complexes with bacterial GroEL while they are in unfolded or partially folded states (Viitanen *et* al., 1992). GroEL has been shown to be an immunodominant antigen and a major immunogen of many bacterial pathogens (Kaufmann, 1991), but generally it does not act in itself as a protective antigen. For example, mycobacterial Hsp60 has been used as an adjuvant (Peterman *et* al., 1993) and as a carrier molecule for conjugated vaccines (Barrios *et* al., 1992; Perraut *et* al., 1993). Previous studies have shown that mice develop both cell-mediated and humoral immune responses to GroEL during the course of infection with *S. typhimurium* (Brown and Hormaeche, 1989; Gupta *et* al., 1996a) and studies performed in our laboratory (Taylor, 1997) have demonstrated that *S. typhimurium* GroEL is highly immunogenic, producing both long-lived T- and B-cell responses.

High anti-GroEL antibody levels have been detected long after *S. typhimurium* infection (day 104) and when infected mice were challenged subcutaneously with GroEL, a significant DTH reaction developed, even at day 104 post-infection. The fact that this protein elicits a significant immune response and induces immunological memory, suggests that *S. typhimurium* GroEL is a potential candidate used for vaccine design.

Phage display

Phage display consists of a peptide or protein, which is displayed on the surface of a phage as a fusion to a protein that is normally found in the phage particle. Most phage display work has used filamentous phage strains M13, fd, and f1 as vectors (reviewed in Smith and Petrenko, 1997), but display systems based on bacteriophage T4 (Efimov *et* al., 1995; Ren *et* al., 1996) and λ (Sternberg and Hoess, 1995; Mikawa *et* al., 1996) have also shown promise. The first phage vectors suitable for surface display were made by Smith (1985) and consisted in displaying foreign peptides or proteins on the surface of filamentous bacteriophage particles via fusion to the phage coat proteins. As Smith and Petrenko (1997) have pointed out, the main applications of phage display systems are: (1) studies of target receptors for affinity-selection of phage-displayed peptides, (2) epitope mapping and mimicking, (3) identification of new receptors and natural ligands, (4) selection of DNA-binding proteins, and (5) epitope and drug discovery.

The potential of phage T7 for display of peptides was reported initially by Houshmand and co-workers (1999). Very recent studies (Sokoloff *et* al., 2000) have used T7 phage as a peptide display system to analyse the interactions of displayed peptides with the innate immune system. They used the icosahedral T7 phage, displaying random peptides at the carboxi-terminus of the phage coat protein, as a model drug and gene delivery vehicle containing peptide ligands.

In the present study, we explored expression of the *S. typhimurium* GroEL protein on the capsid of the T7 bacteriophage, as a strategy for vaccine design against *S. enterica*. The *groEL* fragment from the chromosome of *S. typhimurium* SL1344 was

cloned into the T7 genome such that it produced a fusion protein with the 10B capsid protein of the phage. The integrity of the overexpressed protein and the use of the hybrid phage in immunisation studies are discussed in later chapters of this thesis.

Bacteriophage T7 – structure and assembly

The T7 bacteriophage, with a diameter of approximately 65 nm, contains double-stranded linear DNA in a cavity enclosed by the capsid's polygonal outer shell. Attached to the outer shell are both an external protein projection (tail) and internal proteins (Serwer *et* al., 1997). The capsid shell on the icosahedral T7 phage is composed of 415 copies of the T7 capsid protein (gene 10) arranged as 60 hexamers on the faces of the shell and 11 pentamers at the vertices (Steven and Trus, 1986). Attached at the remaining vertex are the head-tail connector (gene 8), a short conical tail (genes 11 and 12) and six tail fibres (gene 17). The phage assembly process is similar to that of other double-stranded DNA phages (Cerritelli and Studier, 1996), takes place inside the *E. coli* host cell and mature phage are released by cell lysis. The bacteriophage packages DNA by first assembling a DNA-free, tail-free capsid (procapsid) and then drawing the DNA into this structure (Serwer *et* al., 1997). The procapsid shell is made of scaffolding protein (gene 9), capsid protein (gene 10), the head-tail connector and an integral protein structure (genes 13, 14, 15, and 16). The DNA is packaged from linear concatemers, and as the DNA enters the procapsid shell, the scaffolding protein is released and a conformational change occurs in the shell to form the mature particle. Tail and tail fibres attach at the head-tail connector vertex. Unlike the filamentous systems, proteins displayed on the surface of T7 do not need to be capable of export through the periplasm and the cell membrane (Russel, 1991).

Frameshifting in gene 10 of bacteriophage T7 and protein display

In the case of a frameshift, a significant proportion of ribosomes switch reading frames at a specific site in a coding region, thus entering a new frame. The result is that a single mRNA can produce two products, a protein from the normal coding frame and a trans-frame fusion protein (Condron *et* al., 1991). One particular

example of frameshifting is gene 10 of bacteriophage T7, encoding the capsid protein. This gene has normally two products, a major product of 36 kDa, the 10A protein (344 amino acids), and a minor product of 41 kDa, the 10B protein (397 amino acids; Dunn and Studier, 1983). From an analysis of both DNA and protein sequences, it has been concluded that the minor 10B protein was produced by a translational frameshift into the –1 coding frame, at amino acid 341 of 10A, near the end of the coding frame for the major product. Thus, 10B contains most of the sequence of 10A with 52 extra amino acids from the alternate frame added onto the C-terminus (Dunn and Studier, 1983). The frameshift occurs at a frequency of about 10% of that of normal elongation and both products are incorporated into the capsid in about this proportion. In terms of DNA-encoded gene 10 products, with the translational start at base 1, the proposed frameshift site is at base 1019, the 10A stop codon is at base 1032, the 10B stop codon is at base 1187, and the transcriptional terminator is from base 1198 to 1237 (Condron *et* al., 1991).

Functional capsids can be composed entirely of either 10A or 10B, or of various ratios of the proteins. The region of the capsid protein unique to 10B is on the surface of the phage and may be used for phage display (Novagen Inc., USA). The T7 bacteriophage has been modified as a protein display system for this purpose (T7 Select Phage Display System). The T7Select 1-1b Phage Display System has been used over the course of this study for low-copy number display (0.1-1 copies per phage). The vector contains a multiple cloning site between amino acids 348 and 363 of the 10B gene. The coding sequence for the protein to be displayed can be cloned between the *Eco*RI/*Hin*dIII arms of the T7Select 1-1b vector.

To obtain low-copy number display of the protein, the promoter of the capsid gene (ϕ10) and the s10 translation initiation signal (ribosome binding site) have been removed (Figure 3.1). The capsid mRNA is produced from phage promoters located further upstream of the gene, but production of capsid protein is greatly reduced. Target sequence is fused to the C-terminus of the 10B capsid protein near amino acid 348. The natural translational frameshift site within the capsid gene has been removed, so only a single form of capsid protein is made from this vector,

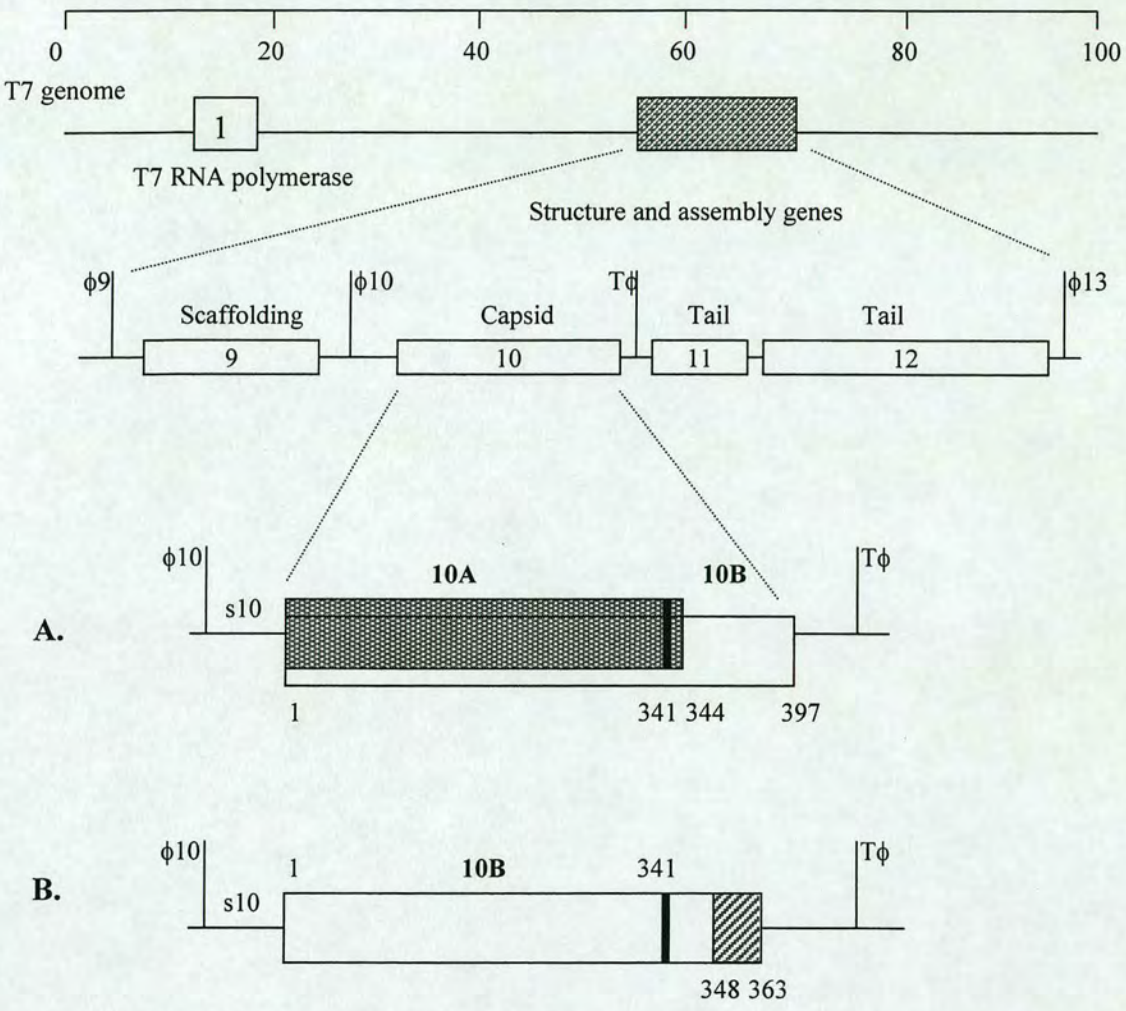

Figure 3.1

The genetic map of T7 and the phage display vector T7Select 1-1b.

The T7 capsid gene (gene 10) is located at position 60 in the T7 genome, within the region of genes coding for proteins involved in the structure and assembly of T7. Capsid protein expression during infection is controlled by a promoter (ϕ10) and terminator (Tϕ) for T7 RNA polymerase, and by translation initiation signals (s10). **A.** In wild-type T7, the capsid protein is normally made in two forms, 10A (344 amino acids), and 10B (397 amino acids), the latter being related by a translational frame-shift at the amino acid 341 within 10A. **B.** The T7Select 1-1b vector used in this study, contains a multiple cloning site, between amino acids 348 and 363 of the 10B gene. The natural translational frameshift site within the capsid gene has been removed and therefore only the truncated 10B form is made from this vector (adapted from inNovations 6 [1996]; Newsletter of Novagen, Inc., USA).

which is the truncated 10B protein. The hybrid T7 phage is grown on a complementing host BLT5615 (Table 2.3) that provides large amounts of the 10A capsid protein from a plasmid, whose expression is controlled by a *lacUV5* promoter. The 10A gene in the complementing plasmid and the capsid gene in the vector have been engineered to minimise any recombination between the genes. For the hybrid T7 phage then, capsids contain mostly 10A protein along with 0.1-1 copies of the fusion protein (*e.g.*, 10B fused at its C-terminal end with *S. typhimurium* GroEL) per virion. The display number from the T7Select 1-1b vector is not strictly fixed, it presumably depends on the ratio of expression of the capsid fusion protein from the vector and the 10A protein from the complementing host (BLT5615), and also on the efficiency of assembly of the fusion protein into the capsid shell (Novagen Inc., USA).

3.1.2 Genetic characterisation of the *S. typhimurium groEL* gene

Previous studies in our laboratory have reported that *S. typhimurium* contains only one homologue of *groEL*, which exists in an operon with *groES*, and by Southern blot analysis the *groESL* locus has been mapped to approximately 93-96 genetic minutes on the *S. typhimurium* chromosome (Taylor, 1997). However, very little information exists in the databases about the genetic sequence of the *S. typhimurium* homologue of GroEL. With regard to these considerations, the present study offers the complete DNA sequence of *S. typhimurium groEL*, which has been initially PCR amplified from *S. typhimurium* SL1344 chromosome, and then cloned in different vectors for overexpression of the protein. Based on the high degree of homology among the *groEL* genes from the related species *E. coli* and *S. typhi* (Figure 3.2), primers have been designed for PCR amplification of the *groEL* fragment from the *S. typhimurium* SL1344 chromosome. Furthermore, the integrity of the GroEL protein expressed from the cloned gene have been verified by Western blot analysis.

3.2 RESULTS

3.2.1 Cloning and overexpression of the *S. typhimurium groEL* gene

Cloning of the *groEL* gene into pBR325

As a first step in cloning and overexpression of the *groEL* gene from *S. typhimurium*, a plasmid pIB1 was constructed (see Table 2.2). This plasmid contains the *groEL* gene from *S. typhimurium* SL1344, cloned between unique *Eco*RI and *Hin*dIII sites within pBR325 (Bolivar, 1978; Blank and Wilson, 1982). Based on the high 93% identity between *groEL* genes from *E. coli* (X07850; Hemmingsen *et* al., 1988) and *S. typhi* (U01039; Lindler and Hayes, 1994), primers were devised to the 5'- and 3'- ends of the *groEL* gene, to include nucleotides 1-18 (GroEL53; Lindler and Hayes, 1994) and 2117-2102 (GroH3TTA; Hemmingsen *et* al., 1988), respectively (Figure 3.2). These primers also incorporated *Eco*RI or *Hin*dIII sites at their 5'- ends respectively (see Table 2.1), for directional cloning into pBR325. Using these primers, the 1.6 kb *groEL* fragment was amplified from the genomic DNA of *S. typhimurium* SL1344 by PCR (Figure 3.3). The product was then restricted with *Eco*RI and *Hin*dIII endonucleases, and cloned into the corresponding sites of pBR325 (see Table 2.2) to form pIB1 (see Figure 3.4).

Following electroporation into DH5α cells, the transformants were grown for 12-16 hours at 37^0C on LB/Ampicillin plates. 71 colonies out of 75 transformants, were found to be Amp^R, Tet^S, Cml^S. As further proof, the pIB1 plasmid was isolated and subjected to digestion with endonucleases (Figure 3.5). After agarose gel electrophoresis, pIB1 digested with either *Eco*RI and *Hin*dIII, gave a unique band of 6.4 kb, and the *Eco*RI/*Hin*dIII restricted pIB1 gave two bands, of approximately 4.7 kb, which is the size of the rest of pBR325, and 1.6 kb, confirming the presence of

Figure 3.2 - The alignment of the *groEL* fragment from *E. coli* (X07850) and the *groEL* fragment from *S. typhi* (U01039) showed 93% identity between the 2 fragments. (Key: "*" represents identical/conserved residues in all sequences of the alignment; numbers on the right indicate nucleotide positions in the DNA sequences). Primers GroEL53 and GroH3TTA were devised as shown.

```
                    GroEL53
                    ------------------------>
E.coligroEL   ATGGCAGCTAAAGACGTAAAATTCGGTAACGACGCTCGTGTGAAAATGCTGCGCGGCGTA 60
S.typhigroEL  ATGGCAGCTAAAGACGTAAAATTCGGTAACGACGCTCGTGTGAAAATGCTGCGCGGCGTA 60
              ************************************************************

E.coligroEL   AACGTACTGGCAGATGCAGTGAAAGTTACCCTCGGTCCAAAAGGCCGTAACGTAGTTCTG 120
S.typhigroEL  AACGTACTGGCAGATGCAGTGAAAGTAACCCTCGGTCCGAAAGGCCGTAACGTGGTTCTG 120
              ************************* ********** ************** ******

E.coligroEL   GATAAATCTTTCGGTGCACCGACCATCACCAAAGATGGTGTTTCCGTTGCTCGTGAAATC 180
S.typhigroEL  GATAAATCTTTCGGTGCGCCGACTATCACTAAAGATGGTGTTTCCGTAGCGCGTGAAATC 180
              ***************** ***** ***** ***************** ** *********

E.coligroEL   GAACTGGAAGACAAGTTCGAAAATATGGGTGCGCAGATGGTGAAAGAAGTTGCCTCTAAA 240
S.typhigroEL  GAGCTGGAAGACAAGTTTGAAAACATGGGCGCGCAGATGGTGAAAGAAGTTGCCTCTAAA 240
              ** ************** ***** ***** ******************************

E.coligroEL   GCAAACGACGCTGCAGGCGACGGTACCACCACTGCAACCGTACTGGCTCAGGCTATCATC 300
S.typhigroEL  GCGAACGACGCTGCAGGCGACGGCACCACCACCGCGACCGTACTGGCGCAGTCCATCATT 300
              ** ******************** ******** ** *********** *** * *****

E.coligroEL   ACTGAAGGTCTGAAAGCTGTTGCTGCGGGCATGAACCCGATGGACCTGAAACGTGGTATC 360
S.typhigroEL  ACCGAAGGCCTGAAAGCCGTTGCTGCGGGCATGAACCCGATGGACCTGAAACGTGGTATC 360
              ** ***** ******** ****************************************

E.coligroEL   GACAAAGCGGTTACCGCTGCAGTTGAAGAACTGAAAGCGCTGTCCGTACCATGCTCTGAC 420
S.typhigroEL  GACAAAGCGGTTGCTGCTGCGGTTGAAGAGCTGAAGGCGCTGTCCGTACCGTGCTCCGAC 420
              ************ * ***** ******** ***** ************** ***** ***

E.coligroEL   TCTAAAGCGATTGCTCAGGTTGGTACCATCTCCGCTAACTCCGACGAAACCGTAGGTAAA 480
S.typhigroEL  TCTAAAGCGATTGCTCAGGTAGGTACTATTTCCGCTAACTCCGACGAAACCGTAGGTAAA 480
              ******************** ***** ** ******************************
```

```
E.coligroEL  CTGATCGCTGAAGCGATGGACAAAGTCGGTAAAGAAGGCGTTATCACCGTTGAAGACGGT 540
S.typhigroEL CTGATCGCGGAAGCGATGGATAAAGTCGGTAAAGAAGGCGTCATCACTGTTGAAGACGGT 540
             ******** ********** ******************** ***** ************

E.coligroEL  ACCGGTCTGCAGGACGAACTGGACGTGGTTGAAGGTATGCAGTTCGACCGTGGCTACCTG 600
S.typhigroEL ACCGGTCTGCAGGACGAACTGGACGTGGTTGAAGGTATGCAGTTTGACCGTGGCTACCTG 600
             ****************************************** ***************

E.coligroEL  TCTCCTTACTTCATCAACAAGCCGGAAACTGGCGCAGTAGAACTGGAAAGCCCGTTCATC 660
S.typhigroEL TCTCCTTACTTCATCAACAAGCCGGAAACTGGCGCAGTAGAACTGGAAAGCCCGTTCATC 660
             ************************************************************

E.coligroEL  CTGCTGGCTGACAAGAAAATCTCCAACATCCGCGAAATGCTGCCGGTTCTGGAAGCTGTT 720
S.typhigroEL CTGCTGGCTGATAAGAAAATCTCCAACATCCGCGAAATGCTGCCGGTTCTGGAAGCCGTT 720
             *********** ****************************************** ***

E.coligroEL  GCCAAAGCAGGCAAACCGCTGCTGATCATCGCTGAAGATGTAGAAGGCGAAGCGCTGGCA 780
S.typhigroEL GCAAAAGCAGGCAAACCGCTGCTGATCATCGCTGAAGATGTTGAAGGCGAAGCGCTGGCT 780
             ** ************************************ *****************

E.coligroEL  ACTGCTGTTGTTAACACCATTCGTGGCATCGTGAAAGTCGCTGCGGTTAAAGCACCGGGC 840
S.typhigroEL ACCCTGGTAGTGAACACCATGCGTGGCATCGTGAAAGTGGCTGCGGTTAAAGCACCGGGC 840
             **    ** ** ************************************************

E.coligroEL  TTCGGCGATCGTCGTAAAGCTATGCTGCAGGATATCGCAACCCTGACTGGCGGTACCGTG 900
S.typhigroEL TTCGGCGATCGTCGTAAGGCGATGCTGCAGGATATCGCTACCCTGACCGGCGGTACCGTA 900
             ***************** ** ***************** ******** ***********

E.coligroEL  ATCTCTGAAGAGATCGGTATGGAGCTGGAAAAAGCAACCCTGGAAGACCTGGGTCAGGCT 960
S.typhigroEL ATCTCTGAAGAGATCGGTATGGAGCTGGAAAAAGCAACCCTGGAAGACCTGGGTCAGGCG 960
             ************************************************************

E.coligroEL AAACGTGTTGTGATCAACAAAGACACCACCACTATCATCGATGGCGTGGGTGAAGAAGCT 1020
S.typhigroELAAACGTGTTGTGATCAACAAAGACACCACCACCATCATTGATGGCGTGGGTGAAGAAGCT 1020
            ******************************** ***** ********************

E.coligroEL GCAATCCAGGGCCGTGTTGCTCAGATCCGTCAGCAGATTGAAGAAGCAACTTCTGACTAC 1080
S.typhigroELGCCATCCAGGGCCGTGTTGCTCAGATCCGTCAGCAGATTGAAGAAGCGACCTCCGACTAC 1080
            ** ****************************************** ** ** ******

E.coligroEL GACCGTGAAAAACTGCAGGAACGCGTAGCGAAACTGGCAGGCGGCGTTGCAGTTATCAAA 1140
S.typhigroELGATCGTGAAAAACTGCAGGAGCGCGTAGCGAAACTGGCAGGCGGCGTTGCGGTAATCAAA 1140
            ** ***************** ***************************** ** ******
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E.coligroEL GTGGGTGCTGCTACCGAAGTTGAAATGAAAGAGAAAAAAGCACGCGTTGAAGATGCCCTG 1200
S.typhigroELGTTGGCGCTGCGACCGAAGTTGAAATGAAAGAGAAGAAAGCCCGCGTTGAAGATGCCCTG 1200
           ** ** ***** ********************** ***** ******************
E.coligroEL CACGCGACCCGTGCTGCGGTAGAAGAAGGCGTGGTTGCTGGTGGTGGTGTTGCGCTGATC 1260
S.typhigroELCACGCGACCCGTGCTGCGGTAGAAGAAGGCGTGGTTGCTGGTGGTGGCGTTGCGCTGATC 1260
           ********************************************** ************
E.coligroEL CGCGTAGCGTCTAAACTGGCTGACCTGCGTGGTCAGAACGAAGACCAGAACGTGGGTATC 1320
S.typhigroELCGCGTTGCTTCTAAAATTGCTGACCTGAAAGGCCAGAACGAAGACCAGAACGTGGGTATC 1320
           ***** ** ****** * *********   ** ****************************
E.coligroEL AAAGTTGCACTGCGTGCAATGGAAGCTCCGCTGCGTCAGATCGTATTGAACTGCGGCGAA 1380
S.typhigroELAAAGTTGCGCTGCGCGCAATGGAAGCTCCGCTGCGTCAGATCGTGCTGAACTGCGGCGAA 1380
           ******** ***** ****************************  ***************
E.coligroEL GAACCGTCTGTTGTTGCTAACACCGTTAAAGGCGGCGACGGCAACTACGGTTACAACGCA 1440
S.typhigroELGAGCCGTCTGTTGTCGCTAACACCGTTAAAGGCGGCGACGGTAACTACGGTTACAACGCA 1440
           ** *********** ************************** ******************
E.coligroEL GCAACCGAAGAATACGGCAACATGATCGACATGGGTATCCTGGATCCAACCAAAGTAACT 1500
S.typhigroELGCAACTGAAGAATACGGCAACATGATCGATATGGGTATCTTGGACCCAACCAAAGTTACC 1500
           ***** ********************** ********* **** *********** **
E.coligroEL CGTTCTGCTCTGCAGTACGCAGCTTCTGTGGCTGGCCTGATGATCACCACCGAATGCATG 1560
S.typhigroELCGTTCTGCGCTGCAATACGCGGCTTCTGTGGCTGGTCTGATGATCACTACCGAGTGCATG 1560
           ******** ***** ***** ************** *********** ***** ******
E.coligroEL GTTACCGACCTGCCGAAAAACGATGCAGCTGACTTAGGCGCTGCTGGCGGTATGGGCGGC 1620
S.typhigroELGTGACCGACCTGCCGAAAAGCGATGCTCCTGATTTAGGCGCTGCTGGCGGCATGGGTGGT 1620
           ** ***************** ******  **** ****************** ***** **
E.coligroEL ATGGGTGGCATGGGCGGCATGATGTAA 1647
S.typhigroELATGGGTGGCATGGGCGGCATGATGTAA 1647
           ***************************
```

GroH3TTA

Figure 3.3

PCR amplification of the *groEL* gene from *S. typhimurium* SL1344 chromosome.

Shown is the PCR product (1.6 kb) obtained by PCR amplification of the *groEL* gene from *S. typhimurium* SL1344 chromosome, using primers to the 5'- (GroEL53) and the 3'- (GroH3TTA) ends of the gene. Figures on the left side of the picture show the size of each band of the molecular marker (lane M) and of PCR product (*groEL*).

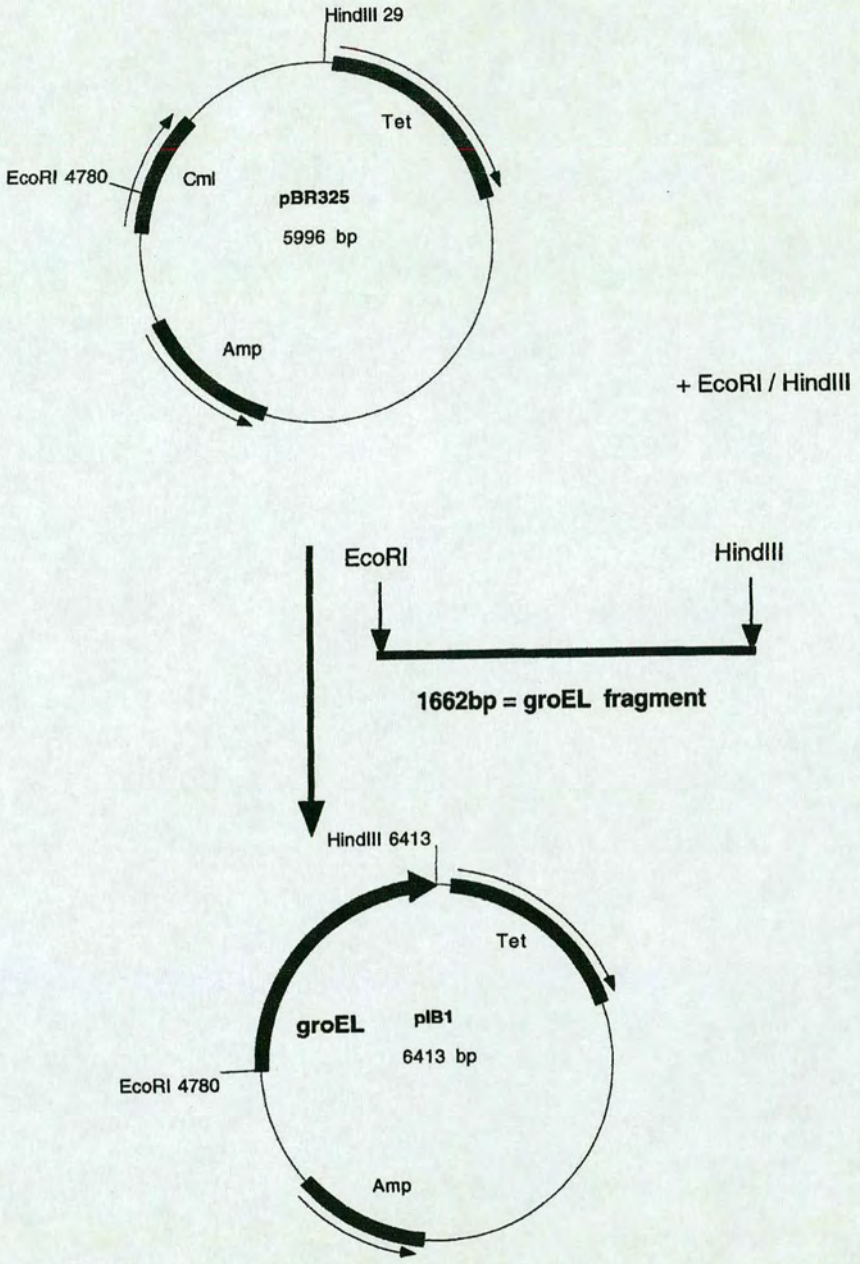

Figure 3.4

Cloning of the *S. typhimurium groEL* gene into pBR325 to generate the plasmid pIB1.

The *groEL* gene (1.6 kb) was amplified from *S. typhimurium* SL1344 chromosome by PCR, then it was restricted with *Eco*RI and *Hin*dIII and cloned into the *Eco*RI and *Hin*dIII unique sites of pBR325 (5.9 kb) to form pIB1 (6.4 kb). Key: *Amp*, gene encoding ampicillin resistance; *Cml*, gene encoding chloramphenicol resistance; *Tet*, gene encoding tetracycline resistance

Figure 3.5 - Restriction analysis of pIB1.

Transformants were verified for the presence of pIB1 by restriction with different endonucleases. Key: lane M, λ/*Hin*dIII molecular weight markers; lane 1, unrestricted pIB1; lane 2, pIB1 restricted with *Eco*RI (6.4 kb); lane 3, pIB1 restricted with *Hin*dIII (6.4 kb); lane 4, pIB1 restricted with both *Eco*RI and *Hin*dIII (4.7 kb and 1.6 kb); lane 5, *groEL* insert as a PCR product (1.6 kb); lane 6, pIB1 restricted with *Bam*HI (6.4 kb); numbers on the left show the size in kilobases of the molecular marker bands.

the *groEL* fragment within pIB1. Interestingly, *Bam*HI restriction of pIB1 has given one linearised fragment (6.4 kb), whereas it was expected that this restriction might give back two fragments. This result will be explained later in terms of the sequencing data obtained for the *S. typhimurium groEL* fragment. Plasmid pIB1 was used over the course of the experiments performed in this study, to avoid further PCR amplifications and the likelihood of inducing point mutations within the *groEL* fragment.

Cloning of the *groEL* gene into pTTQ18

pTTQ18 (4.56 kb) is an expression vector that has been constructed for the regulated expression of genes in *E. coli* (Stark, 1987). Based on the pUC multi-copy plasmids (Vieira and Messing, 1982), the pTTQ18 vector contains a polylinker/*lacZα* region flanked by the strong hybrid *trp-lac* (*tac*) promoter. The promoter comprises the –35 sequence from the *E. coli trp* promoter region fused to the –10 sequence from the *E. coli lacUV5* promoter region (Amann *et* al., 1983; De Boer *et* al., 1983). This strong promoter allows routinely the accumulation of polypeptides at about 15-30% of the total cell protein (Baneyx, 1999). The *tac* promoter is then followed by the ribosome-binding site (RBS) and the translation-initiation codon ATG, followed by a set of unique cloning sites within the polylinker region. Foreign genes can be inserted into the polylinker, within the *lacZα* coding region, to give transcriptional/translational fusions. The plasmid also carries a strong transcription terminator from the *E. coli rrnB* promoter (Brosius *et* al., 1981) downstream from the polylinker region, together with the $lacI^{Q}$ allele of the *lac* repressor gene (Muller-Hill *et* al., 1968). The hybrid promoter is subject to tight regulation by the $lacI^{Q}$ allele, which produces sufficient repressor to prevent expression until addition of the inducer, the non-hydrolysable lactose analogue IPTG (Stark, 1987).

In order to characterise the product of the cloned *groEL* gene from *S. typhimurium* SL1344 chromosome, the *groEL* fragment was cut from the pIB1 vector at its flanking *Eco*RI and *Hin*dIII sites, and cloned within the pTTQ18 polylinker, between *Eco*RI and *Hin*dIII unique sites, downstream of the strong *tac* promoter. This translational in-frame fusion of the *groEL* fragment allowed the production

within the *E. coli* JM109 cells, of large amounts of *S. typhimurium* GroEL protein for studies of the expected size of the product (60 kDa) and of specificity of interaction with anti-GroEL antibodies. The new plasmid was named pIB2 (see Figure 3.6 for pIB2 construction).

Plasmid pIB2 was isolated from the transformants (white colonies) on LB/Ampicillin plates containing X-Gal and IPTG, and then subjected to restriction analysis with various restriction endonucleases, as shown in Figure 3.7.

Overexpression of *S. typhimurium* GroEL from JM109 (pIB2) cells

Before proceeding any further with cloning of the *groEL* fragment into the T7 genome, overexpression of the GroEL protein was examined in *E. coli* JM109 (pIB2). Cells were grown at 37^0C in LB/Ampicillin (100 μg/ml) until an OD_{600} of 0.4. The production of the *S. typhimurium* GroEL protein was then induced by addition of 1 mM IPTG. Induced and non-induced protein samples were solubilised in Laemmli solubilisation buffer (LSB; Laemmli, 1970) by boiling for 5 minutes, and then electrophoresed in a 10% (v/v) polyacrylamide resolving gel (SDS-PAGE), as shown in Figure 3.8A. Subsequently, the *S. typhimurium* GroEL protein was characterised by Western blotting with polyclonal antibodies (1/10,000 dilution) raised against recombinant GroEL from *E. coli* (Sigma, UK). Horseradish peroxidase-conjugated donkey anti-rabbit IgG (SAPU, Scotland), at a dilution 1/10,000, were used as secondary antibodies. Visualisation was performed using the chemiluminescent detection reaction catalysed by horseradish peroxidase (Figure 3.8B).

The immunological reactivity with a protein of apparent molecular mass of 60 kDa, indicated that the *S. typhimurium* GroEL protein was overexpressed, following addition of IPTG. These preliminary cloning and overexpression experiments, proved useful in demonstrating the integrity of the insert at both genetic and protein levels and therefore, this protein could be employed for display on the T7 bacteriophage capsid.

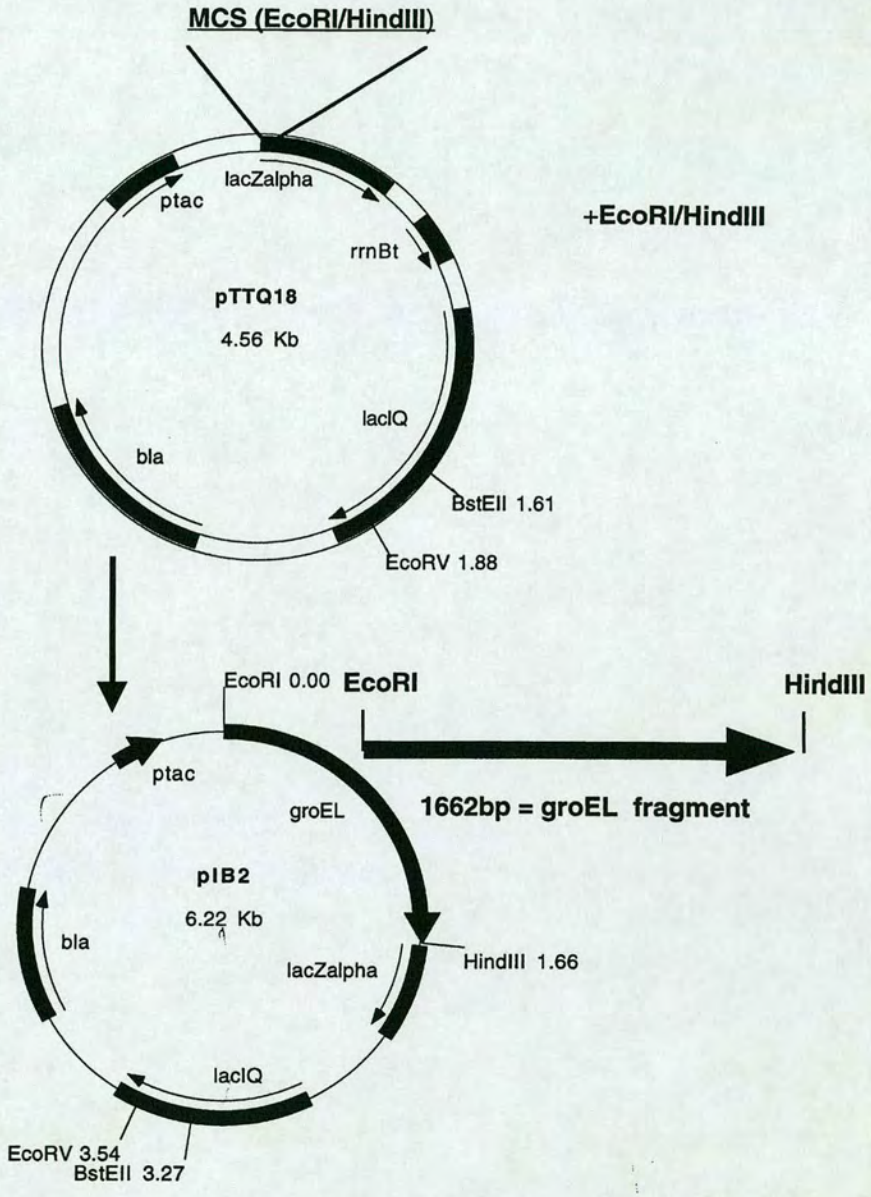

Figure 3.6

Cloning of the *S. typhimurium groEL* gene from pIB1 into pTTQ18 to generate the plasmid pIB2.

The *groEL* gene (1.6 kb) was excised from the pIB1 plasmid with *Eco*RI and *Hin*dIII and then inserted into the plasmid pTTQ18 (4.5 kb), between the *Eco*RI/*Hin*dIII sites within the multiple cloning site to form pIB2 (6.2 kb). Key: *bla*, gene encoding Ampicillin resistance (β-lactamase); MCS, multiple cloning site containing the unique sites, *Eco*RI and *Hin*dIIII, within the *lacZ*α coding region; P*tac*, the hybrid *trp-lac* promoter; *rrnB*, transcription terminator; $lacI^{Q}$ allele of the *lac* repressor gene.

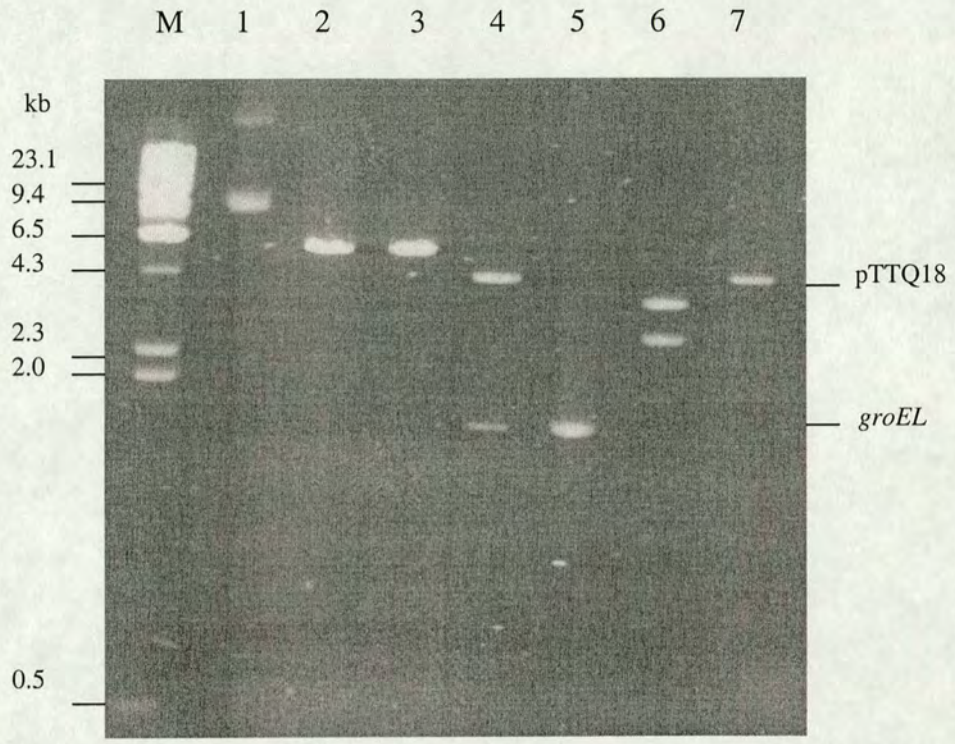

Figure 3.7 - Restriction analysis of pIB2.

JM109 transformants were verified for the presence of the pIB2 plasmid by restriction with different endonucleases. Shown is an 0.8% agarose gel in which restricted and unrestricted DNA samples were resolved and then photographed under UV light. Key: lane M, λ/*Hin*dIII molecular weight markers; lane 1, unrestricted pIB2; lane 2, pIB2 restricted with *Eco*RI (6.2 kb); lane 3, pIB2 restricted with *Hin*dIII (6.2 kb); lane 4, pIB2 restricted with both *Eco*RI and *Hin*dIII simultaneously, which gave rise to a 4.5-kb fragment (pTTQ18), and to the *groEL* insert (1.6 kb); lane 5, *groEL* PCR product (1.6 kb); lane 6, pIB2 restricted with *Eco*RV (as shown in Figure 3.6, pIB2 contains one *Eco*RV site which is located within pTTQ18 inside the $lacI^Q$ gene, and a second putative *Eco*RV site within the *S. typhimurium groEL* fragment); lane 7, pTTQ18 digested at the *Sph*I within the multiple cloning site.

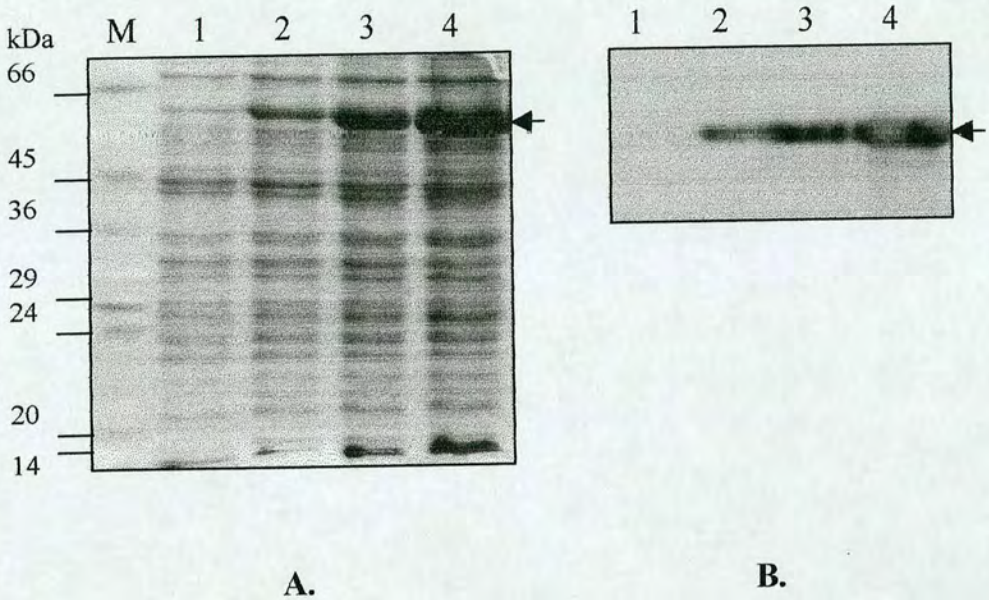

Figure 3.8

Overexpression of *S. typhimurium* GroEL protein from induced JM109 (pIB2) cells.

The *S. typhimurium* GroEL protein was induced in *E. coli* from the *Ptac* promoter of pIB2, by addition of 1mM IPTG. Panel A: un-induced and induced samples were solubilised in 100 µl LSB by boiling for 5 minutes, and then 15 µl from each solubilised sample were resolved on a 10% (v/v) polyacrylamide resolving gel and stained with Coomassie Brilliant Blue. Panel B shows a Western blot of a parallel protein gel, using polyclonal anti-GroEL antibodies for detection. Key: lane M, molecular size marker SDS-70L (Sigma); lane 1, un-induced JM109 (pIB2); lanes 2, 3 and 4, JM109 (pIB2) induced for 30 minutes, 60 minutes and 90 minutes, respectively. The position of GroEL is shown by the arrowheads.

Cloning of *S. typhimurium groEL* fragment into the T7 genome

The procedure for cloning into the T7 vector (Novagen Inc., USA) consists in ligation of the insert between the vector arms provided in the kit, and incubation with an *in vitro* packaging extract. The phage product is subsequently infected into a suitable *E. coli* host (*e.g.*, BLT5615). T7Select 1-1b vector dephosphorylated *Eco*RI/*Hin*dIII arms were therefore used for directional cloning of the *S. typhimurium groEL* fragment. To provide compatibility with the vector arms and obtain expression in-frame with the 10B protein, the *groEL* insert was cut from its flanking *Eco*RI/*Hin*dIII sites within the pIB1 plasmid (see Figure 3.9). Ligation reactions (5.0 µl) were incubated at 16^0C for 16-20 hours and then added directly to T7 Packaging Extracts for *in vitro* packaging of the phage. After incubation at room temperature for 2 hours, packaging reactions were stopped by adding sterile LB medium, and then stored at 4^0C with 20 µl chloroform, until further use. Approximately 5x 10^4 pfu/ml was obtained when the mixture contained insert whilst in contrast, no plaques were observed in the absence of insert.

Amplification of the T7 hybrid phage was then performed (see Chapter 2), to allow display of GroEL on the surface of the phage particles. This also was performed for positive control T7 phage, which expressed an S.tag peptide on the capsid shell. After plate lysate amplification, the titre of the recombinant phage was calculated to be 4.5 x 10^6 pfu/ml whilst 2 x 10^9 pfu/ml were obtained for the positive control.

Identification and analysis of the recombinant T7 bacteriophage

After the T7 recombinant phage had been constructed and amplified, screening of the T7 bacteriophage plaques for the *groEL* insert was performed by *in situ* hybridisation with a ^{32}P-labelled DNA probe. Plasmid pIB1, which contains the *S. typhimurium groEL* insert, was labelled by incorporation of ^{32}P-dCTP into duplex DNA by nick translation, using a Promega kit. The recombinant T7 bacteriophage was plated at approximately 400-500 plaques per 90-mm plate (corresponding to a 10^{-3} dilution of the amplified T7 phage), and an imprint of the pattern of plaques was obtained by layering a nitro-cellulose filter onto the surface of the LC top agar. After denaturation with alkali, the phage DNA was bonded irreversibly to the filter by

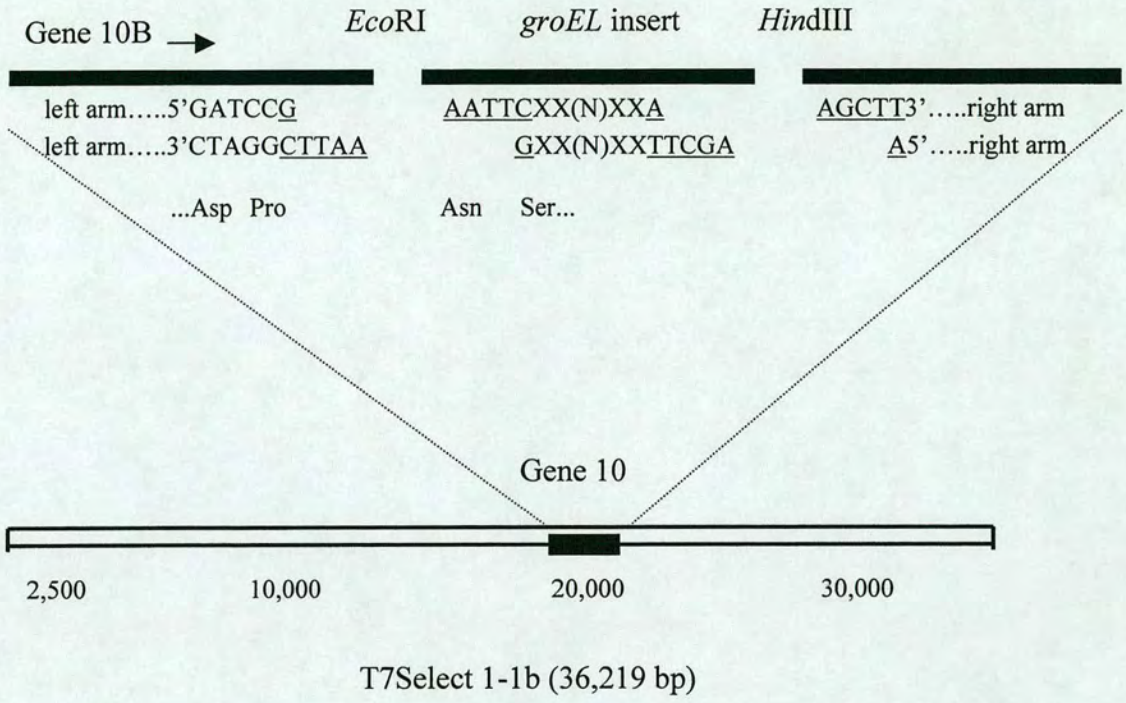

Figure 3.9

The reading frame of the *groEL* insert cloned into *Eco*RI/*Hin*dIII T7Select 1-1b vector arms.

Shown is the insertion of the *groEL* fragment (1.6 kb) between *Eco*RI/*Hin*dIII T7Select 1-1b arms. The approximate position of gene 10 is marked on the T7Select 1-1b genome (36,219 bp). The position of the *groEL* insert fused at the carboxyl-terminus of the 10B protein-encoding fragment is shown. The *Eco*RI and *Hin*dIII restriction sites are underlined. Asp and Pro are encoded on the left arm of the T7 vector, followed by the amino acids Asn and Ser created by an in-frame insertion of the *Eco*RI-cleaved *groEL* fragment.

baking at 80^0C, and then hybridised to the ^{32}P-labelled pIB1 plasmid by overnight incubation at 68^0C in 20 ml of a buffer that contained 6x SSC, 0.5% (w/v) SDS, 50x Denhardt's solution and 25 µl of denatured probe. Excess probe was removed by washing two times in Wash I solution (2x SSC, 0.1% SDS [w/v] in dH_2O) at 68^0C and another two times in Wash II solution (1x SSC, 0.1% SDS [w/v] in dH_2O) at 68^0C, each time for approximately 1½ hours, with rotation. The filters were exposed to an autoradiographic film and hybridising T7 plaques were identified by aligning the film with the original agar plate, as shown in Figure 3.10. As a control for the hybridisation protocol performed in this study, the amplified T7 bacteriophage containing the S.tag peptide was plated on a 90-mm plate at a dilution of approximately 10^{-5} (corresponding to ~200 plaques per plate). However, these phage gave virtually no autoradiography, following the same protocol and using the same ^{32}P-labelled pIB1 plasmid.

Positive T7 recombinant plaques were analysed for the presence of the *groEL* insert, by performing a PCR amplification using a scrape from an individual plaque and primers which flank the multiple cloning site (T7SelectUP and T7SelectDOWN; Table 2.1). Approximately 35 T7 recombinant plaques out of ~ 450, were identified as positive by hybridisation with the ^{32}P-labelled pIB1 plasmid. Following PCR, 10 plaques out of 35, proved positive for the presence of the 1.6 kb *S. typhimurium groEL* insert (Figure 3.11). PCR was also performed for the T7 positive control plaques as above, and the expected 152 bp fragment was obtained (data not shown).

Plaques which were positive for both tests, were purified from 35-ml lysates, as described earlier (see Chapter 2). Essentially, a plug containing a single T7 positive recombinant plaque taken from the agar plate, was added to 35 ml M9LB medium containing Carbenicillin. The T7 were propagated in a BLT5615 host strain at an OD_{600} of 0.5. IPTG (1 mM) was added 30 minutes prior to infection of the T7 phage, to allow expression of the 10A protein from the plasmid. Incubation at 37^0C with shaking was continued until lysis was observed. The clarified lysate was then treated with 5M NaCl to precipitate bacterial proteins, centrifuged, and then the supernatant was mixed with a 50% PEG 8,000 solution to precipitate T7 phage.

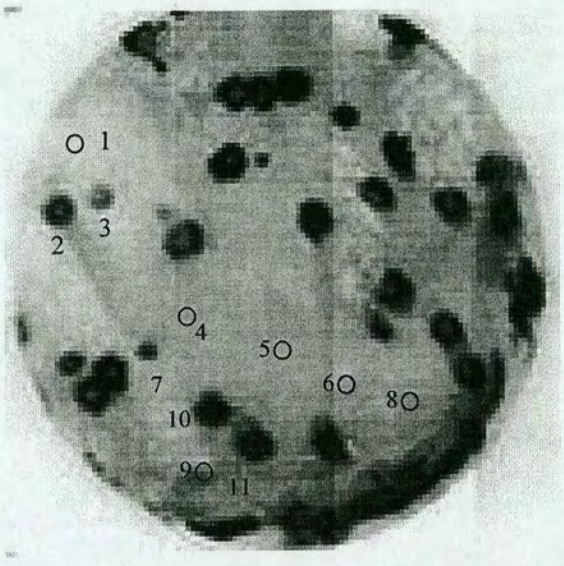

Figure 3.10

In situ hybridisation of the recombinant T7 plaques with ^{32}P-labelled pIB1 plasmid containing the *S. typhimurium groEL* fragment.

Shown is an autoradiographic film, after a 5-hour exposure, of an 90 mm-agar plate containing a 10^{-3} dilution of the amplified T7 recombinant phage after hybridisation with ^{32}P-labelled pIB1. Numbers shown on the film indicate positive and negative plaques subsequently used for PCR amplification of the insert (1.6 kb *groEL* fragment) as shown in Figure 3.11.

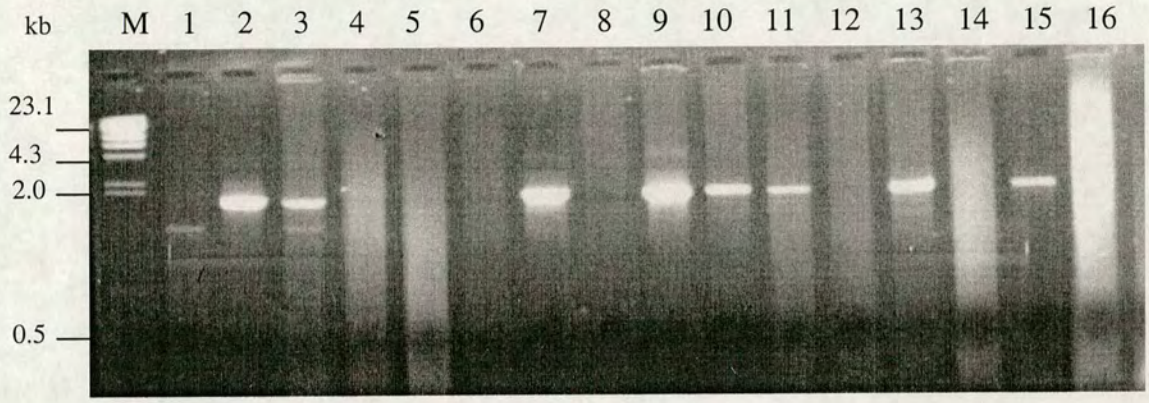

Figure 3.11 - PCR amplification of T7 recombinant plaques.

PCR amplification of the T7 recombinant phage was performed using a scrape from individual plaques. Following PCR with primers T7UP and T7DOWN (see Table 2.1) 10 plaques proved to be positive for the presence of the *groEL* fragment (1.6 kb). Sample numbers correspond to plaques, as shown in Figure 3.10. Key: lane M, molecular weight marker II (Boehringer Mannheim, Germany); lanes 1, 4, 5, 6, 8, 12, 14, 16 are PCR products of negative plaques for *in situ* hybridisation; lanes 2, 3, 7, 9, 10, 11, 13, 15 are PCR products of positive plaques. Only lanes 2, 10, 11, 15 were found to contain a clean PCR product (1.6 kb) following sequencing.

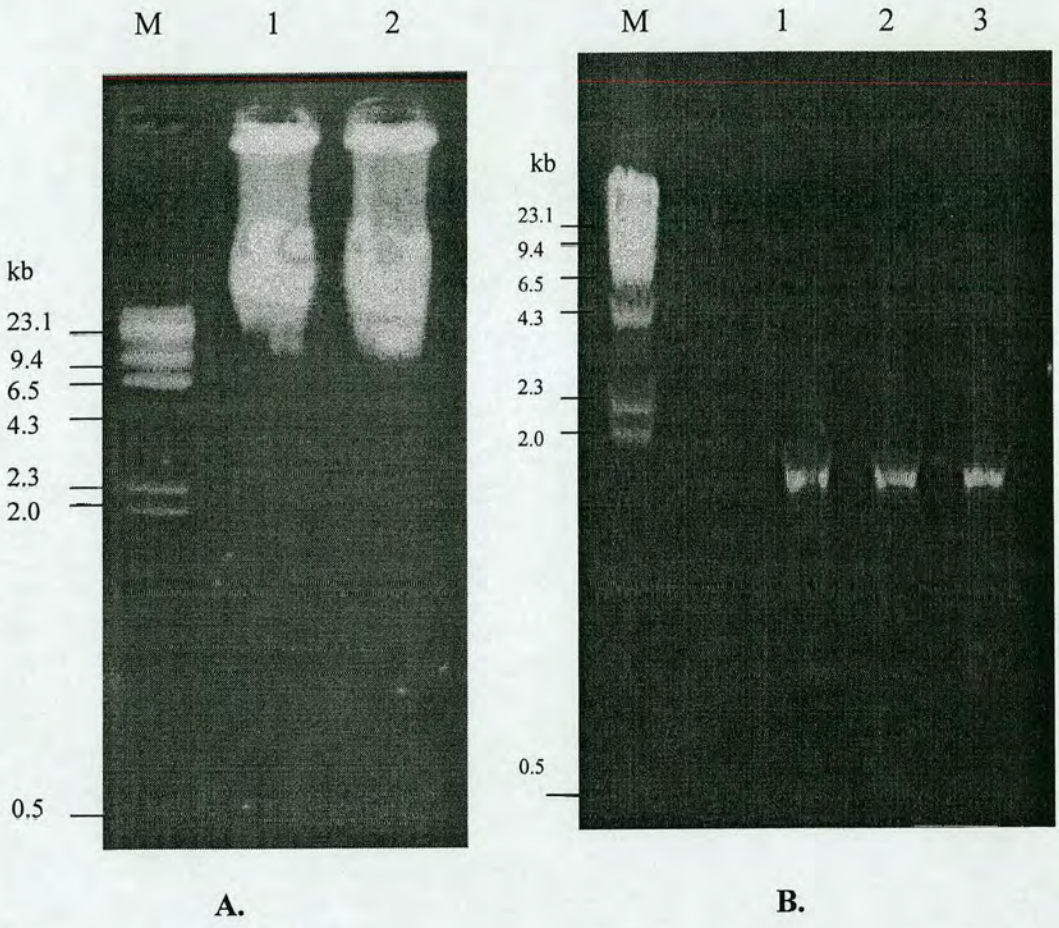

Figure 3.12 - PCR amplification of the T7 recombinant DNA.

A. Recombinant T7 DNA (~ 39,937 bp) was examined before being used as a template in PCR amplification. Key: lane M, λ/*Hin*dIII molecular weight markers; lanes 1, 2, T7 recombinant DNA (100 ng) from the CsCl-purified phage. **B.** PCR products of the T7 recombinant DNA. Key: lane M, λ/*Hin*dIII molecular weight markers; lane 1, PCR product using the primers T7UP and T7DOWN; lane 2, PCR product using the primers T7UP and GroH3TTA; lane 3, PCR product using the primers GroEL53 and T7DOWN.

After centrifugation, phage were resuspended in 10 mM Tris.HCl (pH 8.0) containing 1M NaCl and 1mM EDTA, and the concentrated phage solution was purified by banding in a CsCl step gradient. CsCl layers were made by mixing a stock solution of 62.5% (w/v) CsCl with TE buffer (10 mM Tris.Cl, pH 8.0, containing 1 mM EDTA), and successively denser solutions were underlayered in a SW41 tube using a pipette. The phage solution was layered on top of the 4-step CsCl step gradient, centrifuged and the band containing phage particles was collected with a syringe needle. Purified recombinant T7 phage particles (~ 0.2 ml) were stored stably in the CsCl solution at 4^0C. A plaque assay established their titre as 5x 10^{12} pfu/ml.

The T7 phage were then heat-treated (5 minutes at 65^0C) and ethanol precipitated (see Chapter 2). The DNA was resuspended in 20 µl TE (pH 7.5) and was used for PCR amplification of the *groEL* insert. Figure 3.12A shows the recombinant T7 DNA (~ 40.0 kb) on a 0.5% agarose gel. The amplified fragment from the T7 recombinant DNA prepared previously produced a band of approximatly 1.7 kb fragment (Figure 3.12B).

Sequencing of the *S. typhimurium groEL* fragment cloned into the T7 genome

Purification of large amounts of recombinant T7 phage containing the *groEL* fragment was performed from a 2 litre-lysate (grown as 4 x 500 ml in M9LB medium), as previously described (see Chapter 2). Briefly, 1 mM IPTG was added to four flasks containing 500 ml BLT5615 host cells at an OD_{600} 0.5 for 30 minutes at 37^0C with shaking, to induce 10A protein. Immediately after 10A induction, the host cells were infected with purified recombinant T7 phage containing the *S. typhimurium groEL* fragment (5x 10^{12} pfu/ml; 50 µl per 500 ml culture) and allowed to lyse at 37^0C for 1.5 hours. DNase I was added to each flask at 37^0C for 15 minutes. Bacterial proteins precipitation was facilitated by addition of NaCl and centrifugation. PEG 8,000 was then dissolved in the pooled supernatants to precipitate phage (at 4^0C for 12-16 hours), and was followed by centrifugation. The pellet was washed with 10% PEG 8,000 solution, and the phage were resuspended in 10 mM Tris.HCl (pH 8.0) solution containing 1 mM EDTA and 1 M NaCl prior to

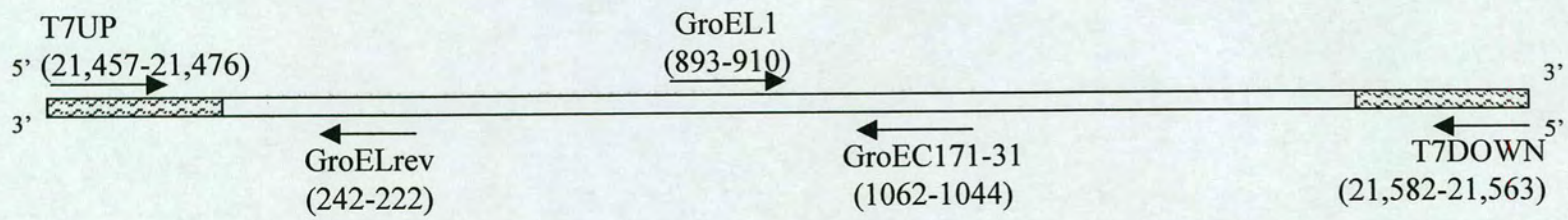

Figure 3.13 - Sequencing strategy for the *S. typhimurium groEL* gene cloned into the T7Select 1-1b genome.

The *groEL* fragment from the recombinant T7 bacteriophage was PCR amplified from the recombinant T7 DNA using the primers T7SelectUP and T7SelectDOWN (their nucleotide positions on the T7 genome are shown in parentheses; see GenBank accession number V01146). Primers used for sequencing (Table 2.1) are shown relative to the organisation of the *E.coli groEL* gene (their nucleotide positions are shown in parentheses; see GenBank accession number X07850) and arrows show the direction of sequencing.

banding in a CsCl gradient (see Chapter 2). Approximately 4 ml purified recombinant T7 phage were obtained with a titre of 5 x 10^{13} pfu/ml. T7 DNA was then prepared from the purified recombinant phage by heat treatment (5 minutes at 65^0C), phenol extraction and ethanol precipitation, and stored as 5 µl-aliquots at – 20^0C until used for PCR or sequencing purposes.

The cloned *groEL* gene was amplified by PCR with primers T7SelectUP and T7SelectDOWN (Table 2.1), which flank the multiple cloning site within the T7Select 1-1b genome. A fragment of approximately 1.7 kb was amplified. This PCR product was purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Germany), and quantified by running 1 µl of the product on a 0.8% (w/v) agarose minigel and comparing the intensity of the band with a control of known concentration (DNA molecular weight marker II, Boehringer Mannheim). 30-90 ng of PCR product was then used as template in cycle sequencing, using a Big Dye Terminator Kit (PE Applied Biosystems, USA).

Figure 3.13 shows a diagram of the strategy followed in order to sequencing the amplified *groEL* gene. Primers used in sequencing (see Table 2.1) are shown relative to the organisation of the *E. coli groEL* gene, upon which they were designed (GenBank accession number X07850). The information generated by the sequencer was collected and analysed using GeneJockeyII (Biosoft) software.

The complete DNA sequence of the entire coding region of *S. typhimurium groEL* gene is shown in Fig. 3.14. The data revealed the absence of a unique *Bam*HI site within the *S. typhimurium groEL* gene, whereas the *E. coli groEL* gene sequence shows the presence of a unique *Bam*HI site at bases 1482-1488 (base pairs numbering relates to the ATG start codon of the *groEL* gene; GenBank accession number X07850). This was confirmed by restriction analysis of the plasmid pIB1 (Table 2.2) with the endonuclease *Bam*HI, which shows one linearised fragment (6.4 kb) after the *Bam*HI restriction (see Figure 3.5) – there is a unique *Bam*HI restriction site within the pBR325 vector. The sequencing data also confirmed that the *S. typhimurium groEL* gene was fused in-frame with the T7 phage 10B protein.

Expression of this fusion protein will be investigated and discussed later in this study.

Figure 3.14 – The nucleotide sequence of the *groEL* gene from *S. typhimurium* SL1344.

Shown is the sequence of the *groEL* gene from *S. typhimurium*, and its corresponding amino acid sequence. The numbers at the left indicate nucleotide position and the numbers at the right indicate amino acid positions in the predicted protein sequence. The nucleotide sequences in bold letters represent the ATG start codon, the TAA stop codon, and, at location 1482, the absence of the *Bam*HI site, found at the same location within the *E. coli groEL* gene (see GenBank accession number X07850).

```
1                                                                   20
atggcagctaaagacgtaaaattcggtaacgacgctcgtgtgaaaatgctgcgcggcgta
M  A  A  K  D  V  K  F  G  N  D  A  R  V  K  M  L  R  G  V

61                                                                  40
aacgtactggcagatgcagtgaaagttaccctcggtccgaaaggccgtaacgtggttctg
N  V  L  A  D  A  V  K  V  T  L  G  P  K  G  R  N  V  V  L

121                                                                 60
gataaatctttcggtgcgccgactatcactaaagatggtgtttccgtagcgcgtgaaatc
D  K  S  F  G  A  P  T  I  T  K  D  G  V  S  V  A  R  E  I

181                                                                 80
gaactggaagacaagtttgaaaacatgggcgcgcaaatggtgaaagaagttgcctctaaa
E  L  E  D  K  F  E  N  M  G  A  Q  M  V  K  E  V  A  S  K

241                                                                100
gcgaacgatgctgcaggcgacggcaccaccaccgcgaccgtactggcgcagtccatcatt
A  N  D  A  A  G  D  G  T  T  T  A  T  V  L  A  Q  S  I  I

301                                                                120
accgaaggcttgaaagccgttgctgcgggcatgaacccgatggacctgaaacgtggtatc
T  E  G  L  K  A  V  A  A  G  M  N  P  M  D  L  K  R  G  I

361                                                                140
gacaaagcggttgctgcggcggttgaagagctgaaagccctgtccgtaccgtgctccgac
D  K  A  V  A  A  A  V  E  E  L  K  A  L  S  V  P  C  S  D

421                                                                160
tctaaagcgattgctcaggtaggtactatctccgctaactccgacgaaaccgtaggtaaa
S  K  A  I  A  Q  V  G  T  I  S  A  N  S  D  E  T  V  G  K

481                                                                180
ctgatcgcggaagcgatggataaagtcggtaaagaaggcgtcatcaccgttgaagacggt
L  I  A  E  A  M  D  K  V  G  K  E  G  V  I  T  V  E  D  G

541                                                                200
accggtctgcaggacgaactggacgtggttgaaggtatgcagtttgaccgcggctacctg
T  G  L  Q  D  E  L  D  V  V  E  G  M  Q  F  D  R  G  Y  L
```

601 220
tctccttacttcatcaacaagccggaaactggcgcagtagagctggaaagcccgttcatc
S P Y F I N K P E T G A V E L E S P F I

661 240
ctgctggctgataagaaaatctccaacatccgcgaaatgctgccggttctggaagccgtt
L L A D K K I S N I R E M L P V L E A V

721 260
gcaaaagcaggcaaaccgctgctgatcatcgctgaagatgttgaaggcgaagcgctggct
A K A G K P L L I I A E D V E G E A L A

781 280
accctggttgttaacaccatgcgtggcatcgtgaaagtggctgctgttaaggcacctggc
T L V V N T M R G I V K V A A V K A P G

841 300
ttcggcgatcgtcgtaaggcgatgctgcaggatatcgctaccctgaccggcggtaccgta
F G D R R K A M L Q D I A T L T G G T V

901 320
atctctgaagagatcggtatggagctggaaaaagcaaccctggaagacctgggtcaggcg
I S E E I G M E L E K A T L E D L G Q A

961 340
aaacgtgttgtgatcaacaaagacaccaccaccatcatcgatggcgtgggcgaagaagct
K R V V I N K D T T T I I D G V G E E A

1021 360
gccatccagggccgtgttgctcagatccgtcagcagattgaagaagcgacctccgactac
A I Q G R V A Q I R Q Q I E E A T S D Y

1081 380
gatcgtgaaaaactgcaggagcgcgtagcgaaactggcaggcggcgttgcggttatcaaa
D R E K L Q E R V A K L A G G V A V I K

1141 400
gttggcgctgcgaccgaagttgaaatgaaagagaagaaagcccgcgttgaagatgccctg
V G A A T E V E M K E K K A R V E D A L

1201 420
cacgcgacccgtgctgcggtagaagaaggcgtggttgctggtggtggcgttgcgctgatc
H A T R A A V E E G V V A G G G V A L I

1261 440
cgcgttgcttctaaaattgctgacctgaaaggccagaacgaagaccagaacgtgggtatc
R V A S K I A D L K G Q N E D Q N V G I

1321 460
aaagttgcgctgcgcgcaatggaagctccgctgcgtcagatcgtgctgaactgcggcgaa
K V A L R A M E A P L R Q I V L N C G E

1381 480
gagccgtctgttgtcgctaacaccgttaaaggcggcgacggtaactacggttacaacgca
E P S V V A N T V K G G D G N Y G Y N A

1441 500
gcaactgaagaatacggcaacatgatcgatatgggtatcct**ggaccc**aaccaaagttacc
A T E E Y G N M I D M G I L D P T K V T

```
1501                                                          520
cgttctgcgctgcagtacgctgcttctgtggctggtctgatgatcactaccgagtgcatg
R  S  A  L  Q  Y  A  A  S  V  A  G  L  M  I  T  T  E  C  M

1561                                                          540
gtgaccgacctgccgaaaagcgatgctcctgatttaggcgctgctggcggcatgggtggt
V  T  D  L  P  K  S  D  A  P  D  L  G  A  A  G  G  M  G  G

1621                    548
atgggtggtatgggcggcatgatgtaa
M  G  G  M  G  G  M  M  *
```

3.3 DISCUSSION

The *groEL* gene from *S. typhimurium* SL1344 chromosome was successfully amplified from the genomic DNA and then cloned within a variety of vectors for different purposes. This allowed verification of protein expression from the amplified and cloned fragment, using plasmid pIB2. Before cloning into the T7 bacteriophage genome, GroEL expression was also confirmed by performing a Western Blot with rabbit anti-GroEL (*E. coli*) antibodies (Sigma, UK). This showed induction of a 60-kDa band corresponding to the *S. typhimurium* GroEL protein, following IPTG induction. The theoretical molecular weight of the *S. typhimurium* GroEL is of approximately 60 kDa.

Purified T7 DNA is easy to obtain in large amounts and has been completely sequenced (GenBank accession number V01146). By cloning of the *S. typhimurium groEL* fragment within the T7Select 1-1b genome, the hybrid T7 phage has been obtained. This phage has the GroEL protein fused in-frame with the 10B protein of the T7 bacteriophage and so should be expressed onto its shell. This will be used in experiments described in the forthcoming chapters.

Very little information exists about the *S. typhimurium* homologue of GroEL. Previous studies in our laboratory have reported that *S. typhimurium* contains only one homologue of *groEL*, which exists in an operon with *groES* (Taylor, 1997). In the present study, the *S. typhimurium groEL* gene has been sequenced. The *S.*

typhimurium SL1344 *groEL* sequence has not been reported previously. The DNA sequence of the open reading frame has revealed 98% identity with the *S. typhi groEL* gene and 93% identity with the *E. coli* gene. Exploration of GenBank for homologues of the predicted protein has identified many members of the Hsp60 family of stress proteins, with a high degree of similarity to GroEL. The amino acid identity range includes 100% for *S. typhi* GroEL, 98% for *E. coli* GroEL and 50% for the human mitochondrial gene product (see Figure 3.15 for an alignment). It cannot be excluded that point mutations may have arisen during the PCR amplification. However, the likelihood of this seems low since no frame-shift errors were identified by sequencing.

As observed elsewhere (Hemmingsen *et* al., 1988), there is a high level of amino acid sequence identity between the prokaryotic and eukaryotic GroEL molecules. With the introduction of only a few gaps, 50% of the residues are identical and many of the differences represent conservative substitutions. However, the eukaryotic GroEL molecules contain an amino terminal sequence of 24-25 amino acids, which is not present in their prokaryotic counterparts.

Based largely on studies of *E. coli* GroEL and mitochondrial Hsp60 in *Saccharomyces cerevisiae*, Hsp60s are believed to reside in the cytoplasm, either in the matrix or stroma of organelles (Craig *et* al., 1993; Soltys and Gupta, 1996). Furthermore, no member of the prokaryotic GroEL family possesses a leader sequence or other recognisable motifs that would suggest a secretory role (Garduno *et* al., 1998), and no such sequence appears within the putative amino acid sequence of *S. typhimurium* GroEL (see Figure 3.14 and Figure 3.15). However, a number of reports indicate an extra-cytoplasmatic location for the GroEL chaperonin. There are studies that report that surface-located Hsp60 mediates adherence of *H. pylori* (Huesca *et* al., 1996), *C. difficile* (Hennequin *et* al., 2001) and *S. typhimurium* (Ensgraber and Loos, 1992) to host cells. Garduno and collaborators (1998) have shown that Hsp60 is surface-exposed during intracellular survival of *L. pneumophila* within host cells and that this molecule also mediates adherence of *L. pneumophila* to HeLa cells. In this respect it has been previously observed that during the early

infection of macrophages by *L. pneumophila*, Hsp60 appears to be released into newly formed phagosomes (Fernandez *et* al., 1996). McLennan and co-workers (1993) have shown that the amino acid sequence of *E. coli* GroEL and other members of the Hsp60 family, share a carboxyl terminal motif, namely the GMR tail, variable in length and which consists mainly of glycine and methionine residues. They have demonstrated that this conserved carboxyl terminal sequence, present also within the putative amino acid sequence of GroEL from *S. typhimurium* (see Figure 3.14 and Figure 3.15), is not required for normal growth and does not extend to adjacent sequences. Moreover, it has been experimentally demonstrated that the carboxyl terminus mediates an interaction of GroEL with lipid layers, and also suggested a lipochaperonin activity for GroEL (Torok *et* al., 1997).

Regarding the three-dimensional structure of prokaryotic and eukaryotic chaperonins, they share a common design in which the substrate is enclosed in a central cavity. Their general structure reveals a radial symmetry, consisting of two rings assembled back to back (Gutsche *et* al., 1999). According to their sequence homologies, chaperonins have been classified into two groups: type I, which exist in bacteria and eukaryotic cellular organelles, that have seven-fold symmetry, and type II, comprising the eukaryotic cytosolic chaperonins, that have eight-folded symmetry (Gutsche *et* al., 1999). Consistent with this remarkable structural similarity, sequences of types I and II chaperonins have significant homologies, especially in the regions corresponding to the equatorial domain. Larger differences appear in the central region of the sequence, corresponding to the GroEL apical domain (Gutsche *et* al., 1999).

Since Buchmeier and Heffron (1990) have observed that mice which are immune to virulent *S. typhimurium* infection mount a strong humoral response to GroEL after they are vaccinated with a live attenuated strain, research has focussed on the requirement for the recognition of GroEL in immunity to *Salmonella* infection. The derived protein sequence of *S. typhimurium* GroEL reported here may facilitate future studies of immune recognition of GroEL by an intracellular pathogen and also help identify epitopes which are recognised during infection.

Figure 3.15 - Alignment of the putative *S. typhimurium* GroEL protein sequence with sequences of GroEL homologues from other organisms.

Key: "*" identical/conserved amino acid residues in all sequences in the alignment; ":" conserved amino acid substitutions; "-" sequence gap; the numbers at the right indicate amino acid positions in the protein sequences. Sequences are taken from: [1], Lindler and Hayes, 1994; [2], Burland *et* al., 1995; [3], Cole *et* al., 1998; [4], Venner and Gupta, 1990; Lotscher and Allison, 1990; [5], Jindal *et* al., 1989.

```
GroELStyphimurium        ------------------------MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGR 36
GroELStyphi[1]           ------------------------MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGR 36
GroELEcoli[2]            ------------------------MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGR 36
GroELMtuberculosis[3]    ------------------------MAKTIAYDEEARRGLERGLNALADAVKVTLGPKGR 35
GroELMouse[4]            MLRLPTVLRQMRPVSRALAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR 60
GroELHuman[5]            MLRLPTVFRQMRPVSRVLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR 60
                                                ** : :  :**  : :*:: ***** **:*****

GroELStyphimurium        NVVLDKSFGAPTITKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATVLA 96
GroELStyphi              NVVLDKSFGAPTITKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATVLA 96
GroELEcoli               NVVLDKSFGAPTITKDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATVLA 96
GroELMtuberculosis       NVVLEKKWGAPTITNDGVSIAKEIELEDPYEKIGAELVKEVAKKTDDVAGDGTTTATVLA 95
GroELMouse               TVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGDGTTTATVLA 120
GroELHuman               TVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGDGTTTATVLA 120
                          *:::: :*:* :*:***::*: *:*:* ::::**::*::** :::: ************

GroELStyphimurium        QSIITEGLKAVAAGMNPMDLKRGIDKAVAAAVEELKALSVPCSDSKAIAQVGTISANSDE 156
GroELStyphi              QSIITEGLKAVAAGMNPMDLKRGIDKAVAAAVEELKALSVPCSDSKAIAQVGTISANSDE 156
GroELEcoli               QAIITEGLKAVAAGMNPMDLKRGIDKAVTAAVEELKALSVPCSDSKAIAQVGTISANSDE 156
GroELMtuberculosis       QALVREGLRNVAAGANPLGLKRGIEKAVEKVTETLLKGAKEVETKEQIAATAAISAG-DQ 154
GroELMouse               RSIAKEGFEKISKGANPVEIRRGVMLAVDAVIAELKKQSKPVTTPEEIAQVATISANGDK 180
GroELHuman               RSIAKEGFEKISKGANPVEIRRGVMLAVDAVIAELKKQSKPVTTPEEIAQVATISANGDK 180
                         :::  **:  :: * **: ::**:  **      *   :      : **   :***  *:
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```
GroELStyphimurium       TVGKLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQFDRGYLSPYFINKPETGAVELE 216
GroELStyphi             TVGKLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQFDRGYLSPYFINKPETGAVELE 216
GroELEcoli              TVGKLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQFDRGYLSPYFINKPETGAVELE 216
GroELMtuberculosis      SIGDLIAEAMDKVGNEGVITVEESNTFGLQLELTEGMRFDKGYISGYFVTDPERQEAVLE 214
GroELMouse              DIGNIISDAMKKVGRKGVITVKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQ 240
GroELHuman              EIGNIISDAMKKVGRKGVITVKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQ 240
                         :* :*::** *** :******::   :  :*:: ***:**:**:* **:   :     ::

GroELStyphimurium       SPFILLADKKISNIREMLPVLEAVAKAGKPLLIIAEDVEGEALATLVVNTMRGIVKVAAV 276
GroELStyphi             SPFILLADKKISNIREMLPVLEAVAKAGKPLLIIAEDVEGEALATLVVNTMRGIVKVAAV 276
GroELEcoli              SPFILLADKKISNIREMLPVLEAVAKAGKPLLIIAEDVEGEALATAVVNTIRGIVKVAAV 276
GroELMtuberculosis      DPYILLVSSKVSTVKDLLPLLEKVIGAGKPLLIIAEDVEGEALSTLVVNKIRGTFKSVAV 274
GroELMouse              DAYVLLSEKKISSVQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV 300
GroELHuman              DAYVLLSEKKISSIQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV 300
                          ::**   *:* :: ::* **      ***:******:****:* *:* ::   :  **

GroELStyphimurium       KAPGFGDRRKAMLQDIATLTGGTVISEE-IGMELEKATLEDLGQAKRVVINKDTTTIIDG 335
GroELStyphi             KAPGFGDRRKAMLQDIATLTGGTVISEE-IGMELEKATLEDLGQAKRVVINKDTTTIIDG 335
GroELEcoli              KAPGFGDRRKAMLQDIATLTGGTVISEE-IGMELEKATLEDLGQAKRVVINKDTTTIIDG 335
GroELMtuberculosis      KAPGFGDRRKAMLQDMAILTGGQVISEE-VGLTLENADLSLLGKARKVVVTKDETTIVEG 333
GroELMouse              KAPGFGDNRKNQLKDMAIATGGAVFGEEGLNLNLEDVQAHDLGKVGEVIVTKDDAMLLKG 360
GroELHuman              KAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGEVIVTKDDAMLLKG 360
                        ******* **  *:*:*  *** *: ** : : **      **:   *:: ** : :: *

GroELStyphimurium       VGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKAR 395
GroELStyphi             VGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKAR 395
GroELEcoli              VGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKAR 395
GroELMtuberculosis      AGDTDAIAGRVAQIRQEIENSDSDYDREKLQERLAKLAGGVAVIKAGAATEVELKERKHR 393
GroELMouse              KGDKAHIEKRIQEITEQLDITTSEYEKEKLNERLAKLSDGVAVLKVGGTSDVEVNEKKDR 420
GroELHuman              KGDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDGVAVLKVGGTSDVEVNEKKDR 420
                         *:   *  *: :* :::: : *:*::***:**:***: ****:* * :::**::*:* *
```

```
GroELStyphimurium       VEDALHATRAAVEEGVVAGGGVALIRVASKIADLKGQNEDQNVGIKVALRAMEAPLRQIV 455
GroELStyphi             VEDALHATRAAVEEGVVAGGGVALIRVASKIADLKGQNEDQNVGIKVALRAMEAPLRQIV 455
GroELEcoli              VEDALHATRAAVEEGVVAGGGVALIRVASKLADLRGQNEDQNVGIKVALRAMEAPLRQIV 455
GroELMtuberculosis      IEDAVRNAKAAVEEGIVAGGGVTLLQAAPTLDELKLEG-DEATGANIVKVALEAPLKQIA 452
GroELMouse              VTDALNATRAAVEEGIVLGGGCALLRCIPALDSLKPANEDQKIGIEIIKRALKIPAMTIA 480
GroELHuman              VTDALNATRAAVEEGIVLGGGCALLRCIPALDSLTPANEDQKIGIEIIKRTLKIPAMTIA 480
                        : **:  ::******:* *** :*::    :  *     *:  * ::   ::: *   *

GroELStyphimurium       LNCGEEPSVVANTVKGGDGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMI 515
GroELStyphi             LNCGEEPSVVANTVKGGDGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMI 515
GroELEcoli              LNCGEEPSVVANTVKGGDGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMI 515
GroELMtuberculosis      FNSGLEPGVVAEKVRNLPAGHGLNAQTGVYEDLLAAGVADPVKVTRSALQNAASIAGLFL 512
GroELMouse              KNAGVEGSLIVEKILQSSSEVGYDAMLGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLT 540
GroELHuman              KNAGVEGSLIVEKIMQSSSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLT 540
                         * * *  :: : :      * :*    : :::  *: ** ** *:**  ** :* *:

GroELStyphimurium       TTECMVTDLPKSDA-PDLGAAGGMGG-MGGMGGMM 548
GroELStyphi             TTECMVTDLPKSDA-PDLGAAGGMGG-MGGMGGMM 548
GroELEcoli              TTECMVTDLPKNDA-ADLGAAGGMGG-MGGMGGMM 548
GroELMtuberculosis      TTEAVVADKPEKEK-ASVPGGGDMGG-MDF----- 540
GroELMouse              TAEAVVTEIPKEEKDPGMGAMGGMGGGMG--GGMF 573
GroELHuman              TAEVVVTEIPKEEKDPGMGAMGGMGGGMG--GGMF 573
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CHAPTER 4

Evidence for co-purification of cellular GroEL with bacteriophage T7

4.1 INTRODUCTION

4.1.1 The T7 particle

Bacteriophages are among the simplest biological entities known, and yet they carry out basic biological processes that occur even in the most complex organisms. Among phages that contain double-stranded DNA, T4 and λ and their relatives have been favoured objects of study. Phage T7 appears to be less complex than T4, and along with its relative T3, is the smallest of the seven T phages originally described. It has a polyhedral head, a small, simple tail, and contains a single piece of double-stranded DNA of molecular weight 25 x 10^6, about one-fourth the size of the DNA from T even phages (Dubin *et* al, 1970). After infection, T7 specifies approximately 30 proteins, which account for virtually all of the coding capacity of T7 DNA (Studier and Maizel, 1969).

4.1.2 Infection cycle of T7 bacteriophage

Initially, T7 was isolated as a phage that grows on *E. coli* B, but T7 and its close relatives grow equally well on *E. coli* K-12 and C strains and on some of the *Shigellae*. A number of *E. coli* strains, as well as other enterobacterial hosts, are nonpermissive for T7. It does not form plaques on fresh natural isolates of *E. coli* because it does not adsorb well to smooth or capsule-containing bacteria, and it can be propagated on *S. typhimurium* strains only if the latter are rough and lack the *hsd*LT restriction system (Molineux, 1999).

The infection cycle of T7 has been described as beginning with the attachment of the tail fibers to the lipopolysaccharide of the *E. coli* outer membrane to initiate adsorption. Following adsorption, the infection process is made irreversible by proteolysis of the internal head proteins, which in turn allows disaggregation and

passage of the internal core structure into the cell. The core proteins enter the periplasm from where they insert into the cytoplasmic membrane, thereby completing a protein channel across the cell envelope for DNA translocation. Only when the transmembrane channel has been formed does DNA leave the phage head, but even then only ~ 850 bp enter the cell immediately. The remainder of the 40-kb genome is normally translocated into the cell as a result of transcription, initially by *E. coli* RNA polymerase and then by T7 RNA polymerase (Molineux, 1999).

4.1.3 T7 proteins

Purified T7 phage particles, when disrupted and electrophoresed on SDS-polyacrylamide gels, give a distinctive pattern of protein bands, which can be visualised by staining or by autoradiography (Studier and Maizel, 1969), and at least 11 different protein chains can be resolved when purified T7 particles are subjected to electrophoresis on SDS-PAGE gels (Studier, 1972). These account for almost half of the coding capacity of T7 DNA. Genes 7, 8, and 10 to 17 specify 10 proteins, and gene 9 specifies a protein found in empty head structures (Studier, 1972).

Gene 10 of bacteriophage T7 specifies the major subunit 10A of the T7 head, a protein having a molecular weight of approximately 36 kDa, which accounts for more than 60% of the mass of the phage particle (Studier and Maizel, 1969), where the T7 phage mass is 7.4×10^{-14} grams (Mazzone, 1998). The second product of this gene, 10B (41 kDa), is produced by frameshifting into the –1 frame near the end of the 10A coding frame (Condron *et* al., 1991). 10B is approximately 10% that of the major subunit 10A (Studier, 1972). Together 10A and 10B represent 415 molecules on the phage capsid. Phages making only 10A or 10B are also viable. 10A-only phages grow like the wild type, but 10B-only phages produce distinctly smaller plaques (Steven and Trus, 1986). This frameshift event appears to be conserved in bacteriophage T3, a divergent relative of T7 (Condreay *et* al., 1989). The gene 10 homologues of other T7-like phages are also thought to make two products; 10B may therefore be important under some conditions of infection (Molineux, 1999).

4.1.4 Time course of T7 protein synthesis

Studier and Maizel (1969) have established patterns of protein synthesis during the growth cycle of wild-type T7 by pulse labelling with radioactive amino acids and SDS-PAGE. They have shown that 80-90% of the phage particles adsorb to their host within 1 minute. Synthesis of most T7-directed proteins begins between 7.5 and 10 minutes after infection and continues until lysis, which begins approximately 25 minutes after infection. Although T4, T5, and SPO1 bacteriophages seem to have complex mechanisms of control over protein synthesis, with T7 the pattern of synthesis seems relatively simple, by comparison (Studier and Maizel, 1969). T7 proteins can be divided into three classes, which are called earliest, early, and late. The late proteins appear approximately 10 minutes after infection and are synthesised until lysis. This class includes proteins of the phage shell, *i.e.* capsid proteins 10A and 10B (Studier and Maizel, 1969), which have been used over the course of this study.

This chapter describes different purification protocols for recombinant T7Select 1-1b bacteriophage (Novagen, Inc., USA), in which the 10B protein of the phage was fused in-frame at its carboxyl terminus with *S. typhimurium* GroEL. In the T7 Select vectors, the natural translational frameshift site within the capsid gene was removed so only the truncated 10B protein is produced. The 10A capsid protein is produced from a plasmid by the *E. coli* BLT5615 host (Table 2.3), in which the recombinant T7 phage is grown (more details have been provided in Chapter2). This recombinant T7 bacteriophage incorporates onto its capsid 0.1-1 copies per virion of the 10B fusion protein. Purification of this hybrid T7 phage was performed prior to immunisation studies on BALB/c mice.

4.2 RESULTS

Analysis of GroEL display on T7 bacteriophage

In Chapter 3, the construction and purification of the recombinant T7 phage, encoding a 10B-GroEL fusion protein, was described. In order to confirm that the fusion protein was displayed on the phage, we purified the phage and examined it by immuno-blotting with anti-GroEL antiserum. For this purpose, recombinant T7 bacteriophage was purified from clarified lysates by precipitation with polyethylene glycol (PEG 8,000) followed by extraction of phage from the PEG pellet in a solution of 10mM Tris.HCl, pH 8.0, containing 1N NaCl and 1 mM EDTA. The concentrated phage solution was subsequently layered on top of four steps of different CsCl density solutions and then banded in the CsCl step gradient, as previously described in Chapters 2 and 3. DNA from a sample of recombinant T7 phage (5 x 10^{13} pfu/ml from 2 litres of cells) was sequenced (see Chapter 3) and proved to have an in-frame fusion of the *S. typhimurium groEL* fragment to the 10B gene of T7. A T7 positive control, containing the S.tag peptide (2 x 10^{9} pfu/ml) was also purified as above and yielded approximately 4 ml of phage, with a titre of 2 x 10^{13} pfu/ml.

The recombinant T7 phage containing the *S. typhimurium groEL* fragment was used to perform a Western blot in order to verify the expression of the 10B-GroEL fusion protein onto the phage capsid. The purified T7 S.tag phage (T7 positive control) was included as a control. Both purified T7 phages were initially solubilised in sample buffer by boiling for 5 minutes and then electrophoresed on a 7.5% (v/v) polyacrylamide resolving gel (SDS-PAGE) as previously described (Laemmli, 1970). Subsequently, proteins were transferred to a nitro-cellulose membrane (Scleicher and Schuell) and then the non-specific binding sites were blocked (see Chapter 2). Polyclonal antibodies raised in rabbit against recombinant *E. coli* GroEL (Sigma, UK) were diluted in a 1% (w/v) solution of non-fat dry milk in T-TBS, and incubated with the membrane for 1 hour at room temperature. After washing, the membrane was incubated with 1% (w/v) milk solution in T-TBS, containing

horseradish-conjugated donkey anti-rabbit IgG (SAPU, Scotland) and binding was visualised using the chemiluminescent detection reaction catalysed by horseradish peroxidase.

As can be seen in Figure 4.1, the purified recombinant T7 phage containing *S. typhimurium groEL* fused with the 10B minor capsid protein of the phage, shows two bands on the Western blot (lane 1): one upper band of approximately 100 kDa, and a lower 60-kDa band corresponding to the size of the purified GroEL protein from *E. coli*. It has been expected that the 10B-GroEL fusion protein would have the size of around 100 kDa, as 10B is 41 kDa in size and *S. typhimurium* GroEL has a predicted size of approximately 60 kDa. The lower band in lane 1 could be explained perhaps, by cleavage of the fusion protein to release GroEL, or by co-association of cellular GroEL as a complex with the fusion protein. However, the presence of the lower band of around 60 kDa in lane 2, which contains S.tag T7 phage (positive control), was not expected and complicated the interpretation.

GroES/EL chaperonins were found to take part in the assembly of the bacteriophage λ, T4, T5, and 186 (Zeilstra-Ryalls *et* al., 1991). GroEL protein from the *E. coli* was shown to act in early steps of phage morphogenesis (Hendrix, 1979) and thus, it may remain attached to the mature T7 phage in the presence or absence of a GroEL fusion protein, through the CsCl purification (lanes 1 and 2, respectively). Conversely, the inability to separate the host GroEL from the T7 *groEL*-containing phage may suggest also that a specific protein-protein interaction may occur due to the natural affinity of GroEL monomers, which normally associate in a multimeric ring.

Pulse chasing of the recombinant T7 phage proteins

In order to help clarify the situation, we decided to examine the *de novo* synthesis of T7 proteins from recombinant T7. When bacteriophage T7 infects *E. coli*, the T7 genes are expressed in an orderly progression (Studier and Maizel, 1969; Studier, 1972). In a normal infection, T7 DNA is transcribed first by the host RNA polymerase (the early region), and then by a T7-encoded RNA polymerase (the late

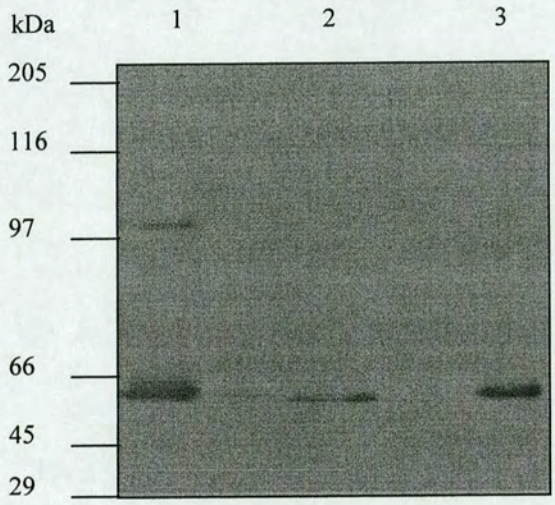


Figure 4.1

Purification and immunoblot analysis of recombinant T7 bacteriophage.

The T7 GroEL-containing bacteriophage were purified using a CsCl step gradient, as described in the text. After purification, phage were solubilised by boiling for 5 minutes in sample loading buffer (1x LSB), then 30 µl was loaded onto a 7.5% (v/v) polyacrylamide resolving gel (16 x 20 cm) and electrophoresed. Shown is a Western blot of the protein gel using polyclonal anti-GroEL antibodies (from Sigma). Key: lane 1, T7 GroEL-containing phage; lane 2, control T7-S.tag phage; lane 3, purified *S. typhimurium* GroEL (0.075 µg); molecular size markers (see Table 2.6) are shown on the left.

region). The class I genes are transcribed by *E. coli* RNA polymerase and include functions to overcome host restriction and to convert the metabolism of the host cell to the production of T7 proteins, the class II genes are the next to be expressed and include functions involved in DNA metabolism, and the class III genes are the last to be expressed (late genes) and include genes for proteins which form part of the phage particle (*i.e.*, gene 10), or function in maturation and packaging of the DNA (Dunn and Studier, 1983). Pulse labelling of T7 phage proteins has been reported previously (Studier and Maizel, 1969; Studier, 1972). We adopted a similar strategy in this study, in order to examine expression of the recombinant 10B-GroEL (~100 kDa) fusion protein from the T7 phage.

An overnight culture of *E. coli* BLT5615 carrying a plasmid from which 10A expression is controlled by a *lacUV5* promoter (see Table 2.3), was diluted approximately 50-fold into LB (with Carbenicillin) and grown with shaking at 37^0C to an absorbance of 0.5 at 600 nm (approximately 4-6 x 10^8 cells/ml). The production of the 10A capsid protein was induced for 30 minutes by addition of 1 mM IPTG. The culture (approximately 10 ml) was then irradiated in an open plastic Petri dish, for 6 minutes with ultraviolet light using a germicidal ultraviolet lamp located at approximately 40 cm above the sample. The cells were returned to a clean flask at 37^0C and shaken for 15 minutes, before infecting with T7. Approximately 5x 10^{10} pfu of T7 phage were added to 1-ml cultures to give a multiplicity of infection of approximately 10 phage particles/cell. The same procedure was performed with a similar number of T7 S.tag phage, as a positive control, and the T7 phage were allowed to infect the *E. coli* cells for 8, 10, 12, 14, and 16 minutes, respectively. Then, each infected (1-ml) culture was mixed briefly with [^{35}S]-methionine (at a final concentration of 15 μCi/ml), and incubated for 2 minutes at 37^0C. The samples were centrifuged for 2 minutes at 12,000 g in a microcentrifuge and then the pellets were resuspended each in 1 ml fresh (cold) LB medium to provide excess unlabelled methionine, and samples were incubated for a further 4 minutes at 37^0C. Samples were pelleted by centrifugation for 5 minutes at 12,000 g, resuspended in LSB, boiled and then loaded onto a 7.5% SDS-PAGE resolving gel. The autoradiogram of

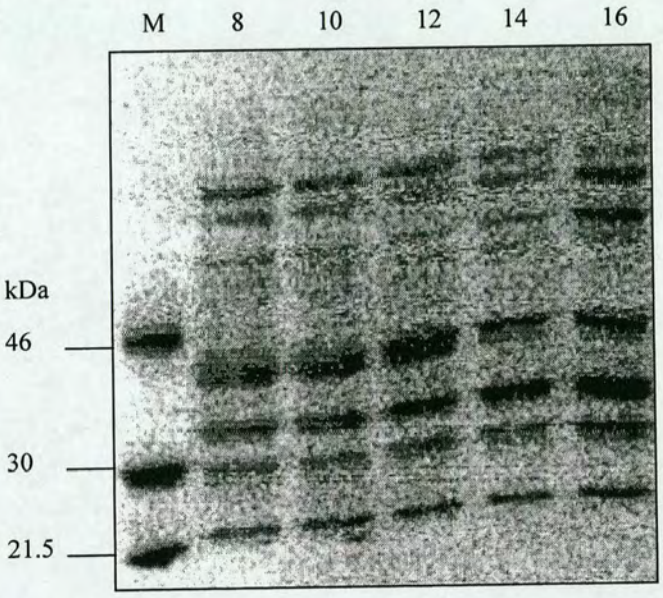


Figure 4.2

Pulse chasing of late T7 proteins from the GroEL-containing bacteriophage.

A culture of *E. coli* BLT5615 grown in LB medium (Carbenicillin supplemented) was irradiated under U.V. light (6 minutes) and then infected with the GroEL-containing hybrid T7 bacteriophage at 37^0C for 8, 10, 12, 14, and 16 minutes, respectively. Then, each culture was pulsed for 2 minutes with [^{32}S]-methionine. Cells were briefly incubated in fresh LB medium, harvested and subjected to electrophoresis on a 7.5% (v/v) polyacrylamide gel (16 x 20 cm) followed by autoradiography, essentially as described by Studier and Maizel (1969). Shown is an autoradiogram of such a 7.5% SDS- polyacrylamide gel through which pulse labelled samples of the GroEL-containing T7 bacteriophage were electrophoresed. Key: lane M, [^{14}C]-methylated protein molecular size markers (from Amersham; see Table 2.7); numbers on top of each lane show the time in minutes at the beginning of each 2-minute pulse. The same protocol was performed for the T7-S.tag control bacteriophage and the autoradiogram showed no difference (data not shown) .

the electrophoresed samples was visualised after 2-day exposure to film at -80^0C (Figure 4.2).

As the 10B phage protein is a late protein of the T7 bacteriophage, it was expected that the fusion protein 10B-GroEL would be visualised after the 10-minute period in which T7 was allowed to infect the host cells. The absence from the autoradiogram of a distinct band, of approximately 100 kDa, may be a consequence of the low display level of the fusion protein per virion, expected to be 0.1-1.0 molecules per phage (Novagen Inc., USA). Pulse labelling experiment under different conditions (using concentrations of 8, 10, and 15 μCi/ml [^{35}S]-methionine), were no more revealing (data not shown). As a result of the initial lack of autoradiographic signals, we decided to try longer exposure periods (from 2 days to 5 days) at -80^0C, and shorter U.V. irradiation periods (from 2 to 4 minutes), in case the ultraviolet light excessively damaged the bacterial cell, such that the phage infection was limited. Longer recovery periods for host cells at 37^0C, after U.V. irradiation, were also examined (15, 25, 35, 45, 55 minutes), but the 15-minute period gave the best result. As a control, radiolabelled T7 phage harvested from the supernatant was assessed for retaining infectivity and proved positive (data not provided).

Extra-purification of the recombinant T7 bacteriophage

The presence of the 60-kDa band on the previously discussed Western blot, namely in lane 1 (see Figure 4.1), may have arisen as a result of association of cellular GroEL. Novagen Inc. describe a further purification for the CsCl-banded T7 phage, which involves adding CsCl 62.6% (w/v) to make the phage solution more dense than is used with the 2:1 CsCl:TE mixture, then floating it up to an interface with an 10-ml upper layer of 1 M NaCl, and centrifuging at 35,000 rpm for 60 minutes in an ultracentrifuge (SW41 rotor). We attempted this procedure in order to determine if the 60-kDa protein would remain associated.

1 ml CsCl-banded T7 phage suspension was subjected to the extra-purification and approximately 0.5 ml of T7 was obtained. This protocol was performed for both the GroEL-containing T7 and for the S.tag-T7 control. Initially, these lysates had titres

of 5 x 10^{13} pfu/ml, and 2 x 10^{13} pfu/ml, respectively and these increased to 5 x 10^{17} pfu/ml following further purification.

The CsCl-banded and further purified phage, were denatured by boiling for 5 minutes in Laemmli solubilisation buffer, and then electrophoresed on a 7.5% (w/v) polyacrylamide gel. After electrophoresis, the proteins from the gel were blotted overnight onto a nitro-cellulose membrane and a Western blot was performed as previously described (see Chapter 2). Figure 4.3 shows the Western blot following exposure to anti-GroEL polyclonal antibodies, anti-immunoglobulin antibodies conjugated to horseradish peroxidase and chemiluminescent detection. For the recombinant CsCl-purified T7, both the upper and lower bands of approximately 100 kDa and 60 kDa were observed, but for the extra-purified phage, the upper band was missing. Moreover, the lower band was less intense, suggesting a possible contamination of the phage with cellular GroEL via direct adhesion to the phage. Therefore, another attempt was made in order to purify the *groEL*-containing T7 bacteriophage.

Purification of recombinant T7 bacteriophage under various conditions

Purification in the presence of a protease inhibitor:

Western blot analysis of CsCl-purified recombinant T7 showed the presence of a 60-kDa band, in addition to the expected 100-kDa band, which represented 10B-GroEL fusion protein (Figure 4.1). In order to address whether the 60-kDa band arose as a result of proteolysis of the 10B-GroEL fusion protein, purification of the phage was carried out in the presence of the protease inhibitor 4-(2-aminoethyl) benzenesulphonylfluoride (AEBSF), a strong serine-protease inhibitor. The polypeptide fused to the bacteriophage 10B capsid protein and exposed on the phage surface, is known to have considerable structural flexibility (Houshmand *et* al., 1999) and may be a target for degradation by proteases. Therefore, the protease inhibitor AEBSF was added to the phage lysate at a final concentration of 0.5 mM, at the stage of purification when the cell lysis occurred, concomitantly with DNase I addition (see Chapter 2), and also to all buffers used throughout the remainder of the purification. The recombinant T7 CsCl-purification in the presence of AEBSF was

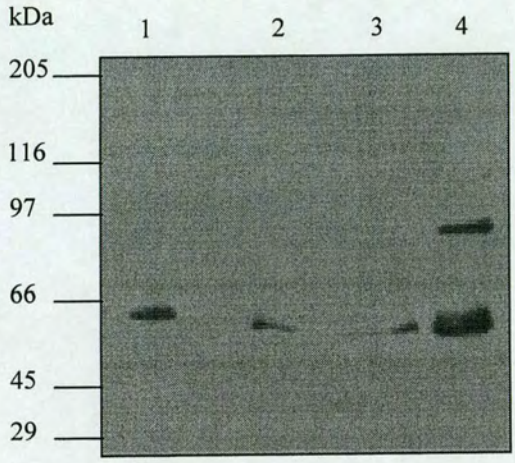


Figure 4.3

Extra-purification of the GroEL-containing T7 bacteriophage.

The GroEL-containing T7 bacteriophage was initially banded through a CsCl step gradient and then further purified through an additional CsCl gradient, as described in the text. 50 µl of purified phage was electrophoresed on a 7.5% (v/v) polyacrylamide gel (7 x 8 cm). Shown is a Western blot of the protein gel using polyclonal anti-GroEL antibodies (Sigma). Key: lane 1, purified *S. typhimurium* GroEL (0.037 µg); lane 2, T7-S.tag positive control; lane 3, T7-GroEL after the second CsCl purification step; lane 4, GroEL-containing T7 bacteriophage after one CsCl purification; numbers shown on the left side correspond to size of protein markers (SDS-6H from Sigma; see Table 2.6).

performed using the single gradient purification, as previously described (2 litre-scale; see Chapter 2). The purified phage (approximately 4 ml) exhibited a titre of 1 x 10^{13} pfu/ml.

Purification in the presence of a protease inhibitor and various other conditions:
Apart from degradation by proteases of the fusion protein, the 60-kDa band on the Western blot may be produced by co-purification of cellular GroEL with the T7 phage. GroEL is known to affect protein folding (Horwich *et* al., 1993; Gaitanaris *et* al., 1994) and to be involved in early stages of phage morphogenesis (Georgopoulos *et* al., 1972; Hendrix, 1979; Tilly and Georgopoulos, 1982; Friedman *et* al., 1984). It has been demonstrated that at least 50% of the soluble proteins in *E. coli* form complexes with GroEL when diluted from denaturant into GroEL-containing solution (Viitanen *et* al., 1992), and that approximately 300 newly translated polypeptides strongly interact with *E. coli* GroEL (Houry *et* al., 1999). The inability to separate the cellular GroEL from the recombinant T7 phage through the CsCl-step gradient purification may suggest a protein-protein interaction, either to the T7 phage itself or/and to the GroEL moiety from the 10B-GroEL fusion protein. It has been demonstrated that GroEL recognises and binds to polypeptides predominantly by hydrophobic interactions (Fenton *et* al., 1994), and that interactions between polypeptides and GroEL are nucleotide dependent: ATP binding and hydrolysis in GroEL control the binding of polypeptide/GroES and the folding/release of the polypeptide (Rye *et* al., 1997). Therefore, for the removal of the co-purifying cellular GroEL, an alternative method described by Thain and co-workers (1996) was employed, which used the addition of ATP and $MgCl_2$ upon phage precipitation.

In order to remove the cellular GroEL attached to the recombinant T7 phage, which gave the 60-kDa band on the Western blot, 1% SDS was added upon phage precipitation. SDS is an anionic detergent, which allows for efficient disruption of most expressed proteins and their ligands, and is included in the T7 Elution Buffer (Novagen Inc., USA) used for biopanning of the recombinant phage.

It is known that the T7 phage is stable to a broad range of harsh treatments, including a solubilising agent such as urea, or a protein denaturant such as the chaotropic agent guanidinium hydrochloride (Novagen Inc., USA). Therefore, proteins that attach to the recombinant T7 phage, such as the cellular GroEL, may be partially denatured with a water-soluble chaotropic agent such as a solution of 4M urea, added upon phage precipitation. Removal of the cellular GroEL attached to the recombinant T7 phage was also tried by addition of 2M guanidinium hydrochloride solution upon phage precipitation with 10% PEG in TE.

The recombinant T7 phage was purified through a CsCl-step gradient from 2 litres of lysate, following the same steps as previously described (see Chapter 2). The entire purification was performed in the presence of the protease inhibitor AEBSF (at a final concentration of 0.5 mM), which was added to the phage lysate and then to all buffers used throughout the purification protocol, as described above. Thus, the 2-litre lysate was split in four after the addition of AEBSF and DNase I, and each 500-ml recombinant T7 lysate followed the same steps for the purification through CsCl gradient, each under different conditions. This experiment was performed for looking at the effect of several factors on purification of the GroEL-expressing T7 phage.

The removal of the putative cellular GroEL was performed by addition of ATP and $MgCl_2$ (each at a final concentration of 5 mM) upon phage precipitation with 10% solution of PEG in TE, based on the concept that binding of ATP by GroEL triggers a conformational shift from a tight binding to a weak binding form of the protein (Badcoe *et* al., 1991; Thain *et* al., 1996). Additionally, AEBSF was added to the phage lysate and to all buffers used, as in the protocol described for the phage purification in the presence of AEBSF. Therefore, from the 2-litre phage lysate, which was split in four 500-ml samples, the 500-ml lysate purified in the presence of 0.5 mM AEBSF, 5 mM ATP and 5 mM $MgCl_2$, approximately 1 ml pure phage was obtained, with a titre of 0.9×10^{13} pfu/ml.

Apart from adding AEBSF (at a final concentration of 0.5 mM) to the T7 phage lysate and to all buffers used throughout the CsCl purification, the recombinant T7 phage was purified in the presence of 1% SDS, added upon phage precipitation with 10% solution of PEG in TE. Therefore, after the 2-litre T7 lysate was split in four, one of the 500-ml lysate was purified in the presence of 0.5 mM AEBSF and 1% SDS. Approximately 1 ml pure recombinant T7 phage was obtained, with a titre of 0.8×10^{13} pfu/ml.

The third 500-ml sample of recombinant T7 lysate, from the 2-litre lysate initially split in four, was purified through CsCl step gradient in the presence of AEBSF, as previously indicated. Urea was added to a final concentration of 4M upon phage precipitation with 10% PEG in TE. After pulling out the putative phage-containing band present above the 2:1 layer in the CsCl step gradient, the phage proved not to be viable and no titre could be established.

The last 500-ml sample from the 2-litre recombinant T7 lysate split in four, was purified through CsCl step gradient in the presence of AEBSF as previously described, and guanidinium hydrochloride was added upon phage precipitation, at a final concentration of 2M. After the ultracentrifugation step, no phage band was observed and after pulling out approximately 1 ml from where the phage band was expected to be situated, no plaques were obtained, which is contradictory to the Novagen technical recommendations.

The T7 recombinant phage purified under the conditions described above were solubilised by boiling in LSB, electrophoresed on a 7.5 % (v/v) polyacrylamide gel, blotted overnight onto a nitro-cellulose membrane, and Western blotted, as previously indicated (see Chapter 2). Figure 4.4 illustrates the Coomassie-stained protein gel (Panel A), and the Western blot (Panel B) performed using polyclonal anti-GroEL antibodies and horseradish peroxidase/ECL detection, as previously described. Figure 4.4A shows faint patterns of the T7 proteins (lanes 3, 4, 5, 6, 7), and of *S. typhimurium* GroEL (lanes 1 and 2) purified using a sucrose density gradient protocol. Use of a Silver stain protocol may circumvent this limitation by

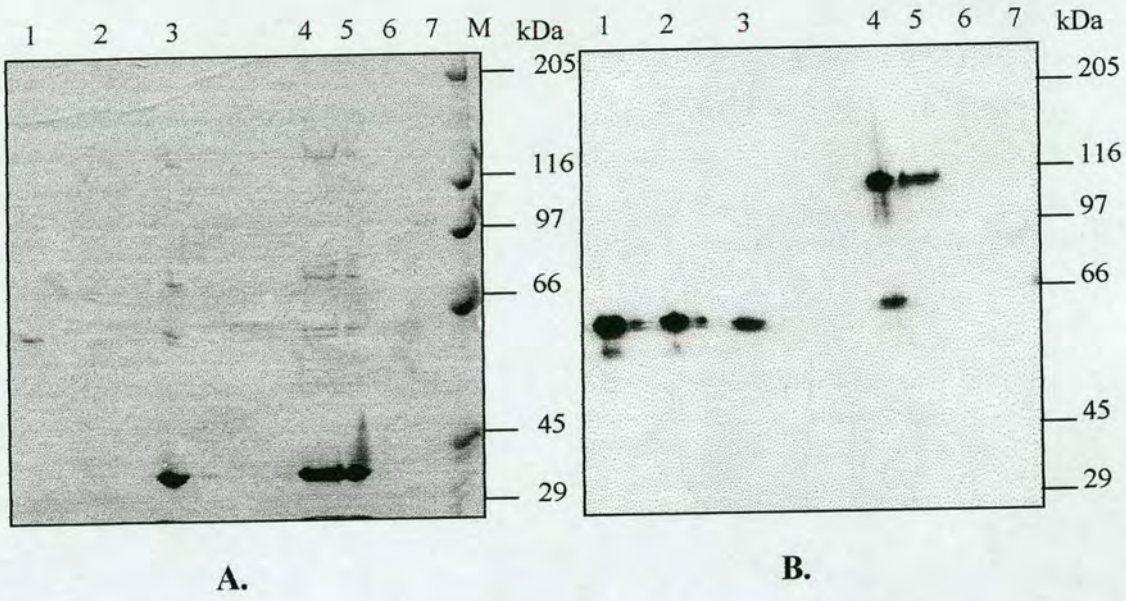


Figure 4.4

Purification of GroEL-containing T7 phage under various conditions.

A. The recombinant T7 bacteriophage was purified through a CsCl step gradient (2L-scale) as previously described. A protease inhibitor (AEBSF) was added at a final concentration of 0.5 mM into the cell lysate and in all buffers used to resuspend the phage throughout the course of purification. After each purification, 50 µl purified phage was loaded onto a 7.5% (v/v) polyacrylamide resolving gel (16 x 20 cm) and stained with Coomassie Brilliant Blue. **B.** Shown is a Western blot of an equivalent gel, using polyclonal anti-GroEL antibodies and peroxidase/ECL detection, as described previously (see Chapter 2). Key: lane M, molecular size markers (SDS-6H; see Table 2.6); lane 1, purified *S. typhimurium* GroEL (0.37 µg); lane 2, purified *S. typhimurium* GroEL (0.075 µg); lane 3, purified recombinant T7 in the presence of AEBSF; lane 4, purified recombinant T7 in the presence of AEBSF and precipitated in the presence of 5 mM ATP and 5 mM $MgCl_2$; lane 5, purified recombinant T7 in the presence of AEBSF and precipitated in the presence of 1% SDS; lane 6, purified recombinant T7 in the presence of AEBSF and precipitated in the presence of 4M urea; lane 7, purified recombinant T7 in the presence of AEBSF and precipitated in the presence of 2M guanidinium hydrochloride.

providing higher resolution and by detecting nanogram quantities of proteins (Blum *et* al., 1987). The intense band visible on the Coomassie-stained gel (lanes 3, 4, 5) is thought to represent the major capsid protein (10A), with a size of 36 kDa (Dunn and Studier, 1983). On the gel, this band is not visible for the recombinant T7 phage purified in the presence of 0.5 mM AEBSF and 4M urea, or 0.5 mM AEBSF and 2M guanidinium hydrochloride (lanes 6 and 7), and may indicate that the T7 phage is not stable when purified under these conditions, finding confirmed by the lack of viability for these purified phage. From Figure 4.4B, it can be seen that the Western blot in which the recombinant T7 phage was purified in the presence of the protease inhibitor AEBSF showed a very intense 60-kDa band and also a faint 100-kDa band (lane 3). This suggests that the treatment with a strong protease inhibitor, such as AEBSF, does not eliminate the presence of the 60-kDa protein shown on the Western blot, and therefore this protein is unlikely to result from cleavage of the 10B-GroEL fusion protein by proteases. This suggests that cellular GroEL remains attached to the recombinant T7 phage and co-purifies with it. The action of proteases which are insensitive to AEBSF cannot be ruled out, however. Lane 4, which contains the recombinant T7 phage purified in the presence of AEBSF, ATP and Mg^{2+}, showed two distinct bands (60-kDa and 100-kDa) on the Western blot, suggesting that the ATP/Mg^{2+} treatment does not remove the contaminating GroEL efficiently. However, lane 5, which contains the recombinant T7 phage purified in the presence of AEBSF and 1% SDS, showed a single 100-kDa band, suggesting that the 1% SDS treatment introduced upon phage precipitation with 10% PEG solution, can eliminate association of phage with the 60-kDa GroEL from cellular sources or from degradation.

Purification of the recombinant T7 phage in the presence of a protease inhibitor and 1% SDS:

As a further test for co-association of the 60-kDa band, purification of T7 phage was also examined in the presence and absence of 1% SDS and the alternative protease inhibitor phenylmethyl-sulphonyl fluoride (PMSF). Purification and Western blot analysis was carried out as before.

The T7 recombinant phage (GroEL-containing) was purified through the CsCl step gradient from 2 litres of lysate (see Chapter 2) obtained by infection of *E. coli* BLT5615 cells with the previously amplified T7 phage (with a titre of 15 x 10^{10} pfu/ml). The T7 lysate was split in four 500-ml lysates and each 500-ml lysate was purified under the following conditions: in the presence of 0.5 mM PMSF, in the presence of 0.5 mM PMSF and 1% SDS, in the absence of PMSF, and in the presence of 1%SDS and absence of PMSF. The protease inhibitor was added upon lysis and was incorporated in all buffers used throughout the purification protocol, and the SDS was added only upon phage precipitation, as described above. Approximately 0.8 ml purified phage were obtained in each case. Their titres were established as follows: the recombinant T7 phage purified in the presence of 0.5 mM PMSF and 1% SDS had a titre of 0.7 x 10^{13} pfu/ml, the T7 phage purified in the presence of 0.5 mM PMSF (without addition of 1% SDS) had a titre of 0.94 x 10^{13} pfu/ml, the T7 phage purified in the absence of 0.5 mM PMSF and 1% SDS had a titre of 0.95 x 10^{13} pfu/ml, and the T7 phage purified in the presence of 1% SDS and with no addition of 0.5 mM PMSF had a titre of 1 x 10^{13} pfu/ml. In parallel, the T7 positive control phage (S.tag-containing), with a titre of 2 x 10^{9} pfu/ml, was used to infect host cells and was then purified from a 1 litre of lysate, through the CsCl step gradient. The lysate was split in two 500-ml samples which were processed as described above, in the presence and absence of the protease inhibitor PMSF (added at a final concentration of 0.5 mM). Approximately 1 ml purified phage were obtained in each case. The T7 positive control phage purified in the absence of PMSF had a titre of 2 x 10^{13} pfu/ml and the T7 positive control phage purified in the presence of 0.5 mM PMSF had a titre of 1.5 x 10^{13} pfu/ml.

The purified T7 phage were boiled for 5 minutes in LSB, loaded on a 7.5% (v/v) polyacrylamide gel, and then electrophoresed. After blotting onto a nitro-cellulose membrane, a Western blot was performed with polyclonal anti-GroEL antibodies, as previously described (see Chapter 2). Figure 4.5 shows the Silver-stained protein gel (Panel A) and the Western blot (Panel B). The T7 GroEL-containing phage purified in the presence of 0.5 mM PMSF, but without addition of 1% SDS upon phage precipitation (lane 7), gave two distinct bands on the Western blot, sized 60 kDa and

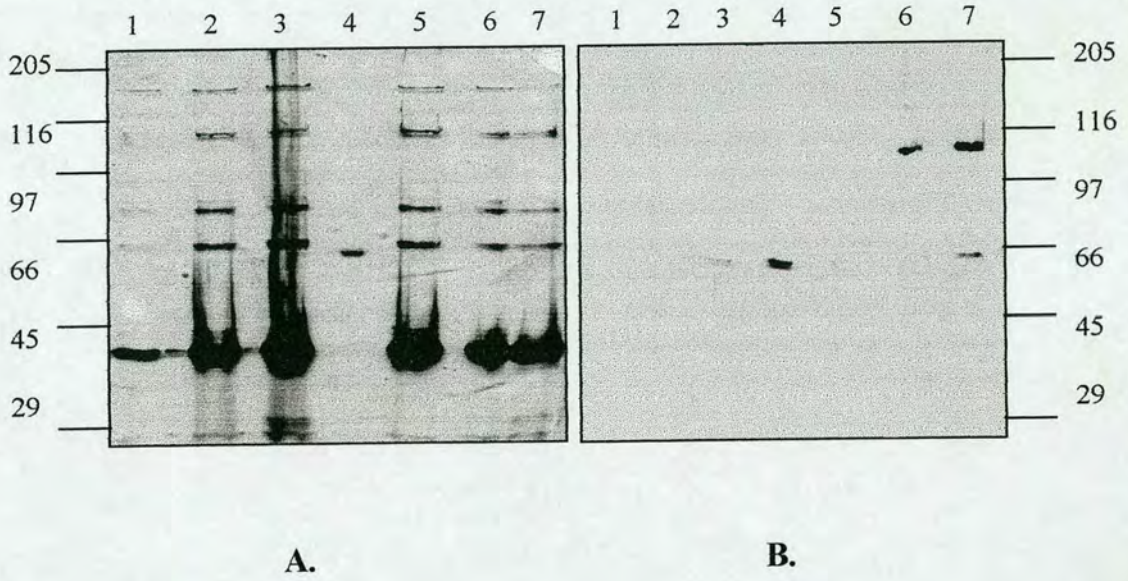


Figure 4.5

Purification of the GroEL-containing and positive control T7 phage.

A. The T7 GroEL-containing and the T7 positive control (S.tag-containing) bacteriophage were purified through a CsCl step gradient (2L-scale), as described in the text. After each purification, 30 µl from each phage were loaded on a 7.5% (v/v) polyacrylamide gel (16 x 20 cm), electrophoresed and the gel was stained using the Silver stain protocol. **B.** Shown is a Western blot of an equivalent gel using polyclonal anti-GroEL antibodies and peroxidase/ECL detection, as described previously (see Chapter 2). Key: lanes 1, 2, and 3 contain purified T7 phage in the absence of any protease inhibitor, as follows: lane 1, T7-S.tag phage; lane 2, T7-GroEL phage precipitated in the presence of 1% SDS; lane 3, T7-GroEL phage precipitated in the absence of SDS; lane 4, purified *S. typhimurium* GroEL (0.075 µg); lanes 5, 6, and 7 contain T7 phage purified in the presence of the protease inhibitor PMSF, as follows: lane 5, T7-S.tag phage; lane 6, T7-GroEL phage precipitated in the presence of 1% SDS; lane 7, T7-GroEL phage precipitated in the absence of SDS; numbers shown on the left side correspond to size of protein markers (SDS-6H from Sigma; see Table 2.6).

100 kDa, suggesting that the 60-kDa band corresponded to cellular GroEL attached to the phage, rather than to the action of proteases. However, the recombinant phage purified in the absence of both the protease inhibitor and SDS (Panel B, lane 3) gave a single 60-kDa band, suggesting that the presence of the protease inhibitor prevented the action of proteases on the fusion protein displayed on the phage capsid. The same recombinant T7 phage purified in the presence of 0.5 mM PMSF and precipitated with 10% PEG solution containing 1% SDS (lane 6), gave a single 100-kDa band on the Western blot, corresponding to the 10B-GroEL fusion protein, indicating that the contaminating cellular GroEL was disrupted efficiently from the phage through the SDS treatment, and also that the addition of the protease inhibitor preserved the integrity of the fusion protein. The absence of bands for the S.tag-containing T7 phage purified in the absence and presence of PMSF (Panel B, lanes 1 and 5) strengthens the conclusion that the 60-kDa band present on Western blot is mainly the result of cellular GroEL associated to the 10B-GroEL fusion protein displayed on the recombinant T7 phage, and not only to T7 proteins.

Purification of the AhpC-containing T7 bacteriophage

Previous studies performed in our laboratory (Francis *et* al., 1997; Taylor *et* al., 1998) established that the *ahp* locus of *S. typhimurium* is a macrophage-induced locus, is not essential for *S. typhimurium* virulence, and that an immune response is elicited against the AhpC polypeptide by both humoral and cellular arms of the immune system during the course of *Salmonella* infection. The *ahp* locus from *S. typhimurium* is a bicistronic locus, which encodes *ahpC* and *ahpF* (Tartaglia *et* al., 1990). Work performed in our laboratory produced a hybrid T7 bacteriophage by cloning a 505-bp fragment from the *S. typhimurium ahpC* gene (166-729 bp; GenBank accession number J05478) between the *Eco*RI/*Hin*dIII arms of the T7Select 1-2a phage (Novagen Inc., USA). The value of the AhpC-containing T7 bacteriophage in this study was to establish the mechanism through which GroEL co-purified with the T7 bacteriophage. This allowed distinguishing between cellular GroEL that stuck to the 10B-GroEL fusion protein, and GroEL which bound to other phage proteins. This AhpC-containing T7 phage was also used in immunisation

studies on BALB/c mice, in parallel with the *S. typhimurium* GroEL-containing T7 phage.

In order to undertake analysis of GroEL binding to the AhpC-containing T7 bacteriophage, it was first of all necessary to validate the DNA sequence of the insert. For this purpose, the fragment was amplified using primers T7UP and T7DOWN (see Table 2.1), and DNA from the T7-AhpC phage (following phenol/chloroform extraction of CsCl-purified phage). Primers T7UP, T7DOWN and SPHI-ANTI739 (Table 2.1) were used for sequencing (see Figure 4.6). The sequencing data confirmed that the *ahpC* fragment, inserted into the T7Select 1-2a genome, is fused in-frame with the 10B gene and that no point mutations were introduced by performing the PCR amplification (see GenBank J05478 for the *S. typhimurium ahpC* sequence).

Having validated the insert sequence, the T7-AhpC bacteriophage was purified through a CsCl step gradient, as previously described (see Chapter 2). The 2-litre clarified lysate was split in four 500-ml lysates and separately processed through the CsCl step gradient purification as follows: in the presence of 0.5 mM PMSF and 1% SDS, in the presence of 0.5 mM PMSF and without addition of 1% SDS, in the absence of both 0.5 mM PMSF and 1% SDS, and in the absence of 0.5 mM PMSF but under addition of 1% SDS. Approximately 0.8 ml purified phage were obtained in each case. The titres of the resulting phage were: 0.5×10^{13} pfu/ml for the phage purified in the presence of 0.5 mM PMSF and 1% SDS, 0.5×10^{13} pfu/ml for the phage purified in the presence of 0.5 mM PMSF and without addition of 1% SDS, 0.5×10^{13} pfu/ml for the phage purified without addition of 0.5 mM PMSF or 1% SDS, and 0.5×10^{13} pfu/ml for the phage purified in the presence of 1% SDS (without addition of 0.5 mM PMSF), respectively.

The recombinant AhpC-containing T7 phage was used as a control system to compare with the GroEL-containing T7 bacteriophage. The presence of cellular GroEL was tested by purifying the AhpC-containing T7 phage under various conditions and performing a Western blot with polyclonal anti-GroEL antibodies, as

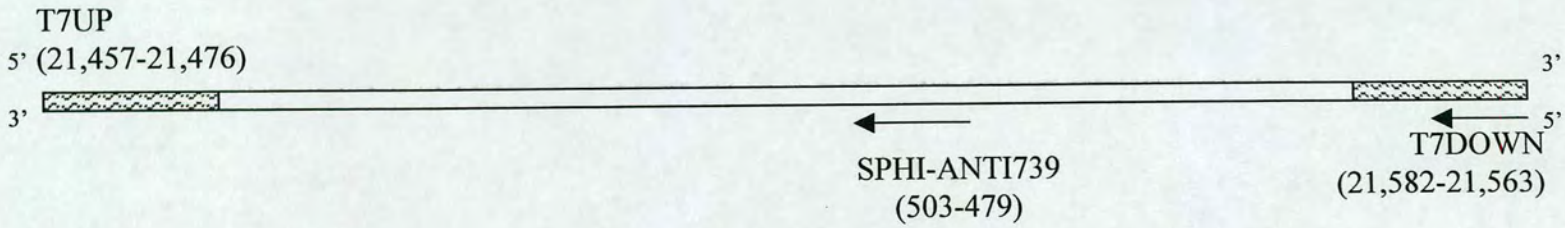


Figure 4.6 - Sequencing strategy for the *S. typhimurium ahpC* fragment cloned into the T7Select 1-2a genome.

The *ahpC* fragment from the recombinant T7 bacteriophage was PCR amplified from the recombinant T7 DNA using the primers T7SelectUP and T7SelectDOWN. The resulting fragment (~600 bp) contained the 505-bp *S. typhimurium ahpC* fragment and was sequenced using primers T7UP, T7DOWN and SPHI-ANTI739 (see Table 2.1). Primers positions are shown in parentheses, relative to the organisation of the *S. typhimurium ahpC* gene (SPHI-ANTI739), and to the T7 genome (T7UP, T7DOWN). Arrows show the strands sequenced and the direction of sequencing. Shaded areas corresponding to portions of T7 DNA containing the T7 primers.

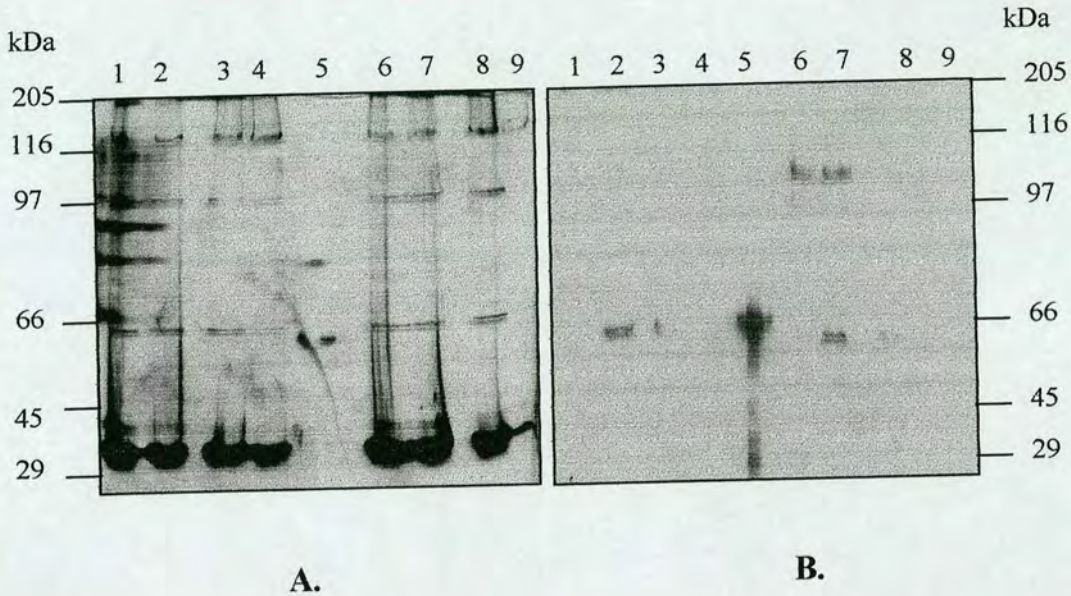


Figure 4.7

Purification of the AhpC-containing and GroEL-containing T7 phage under various conditions prior to being used in immunisation studies on BALB/c mice.

A. The AhpC-containing and GroEL-containing T7 bacteriophage were purified through a CsCl step gradient (2L-scale), as described in the text. After each purification, purified phage were loaded on a 7.5% (v/v) polyacrylamide resolving gel (16 x 20 cm), electrophoresed and stained using the Silver stain protocol. **B.** Shown is a Western blot of an equivalent protein gel analysed using polyclonal anti-GroEL antibodies and peroxidase/ECL detection, as previously described (see Chapter 2). Key: lanes 1, 2, 3, and 4 contain purified T7 phage in the absence of a protease inhibitor (PMSF), as follows: lane 1, T7-GroEL phage precipitated with 1% SDS; lane 2, T7-GroEL phage precipitated in the absence of SDS; lane 3, T7-AhpC phage precipitated in the absence of SDS; lane 4, T7-AhpC phage precipitated with 1% SDS; lane 5, purified *S. typhimurium* GroEL (0.37 μg); lanes 6, 7, and 8 contain purified T7 phage in the presence of the protease inhibitor PMSF, as follows: lane 6, T7-GroEL phage precipitated with 1% SDS; lane 7, T7-GroEL phage precipitated in the absence of SDS; lane 8, T7-AhpC phage precipitated in the absence of SDS; lane 9, T7-AhpC phage precipitated with 1% SDS; numbers shown on the sides correspond to size of protein markers (SDS-6H; see Table 2.6).

described previously. The purified phage were boiled for 5 minutes in LSB, loaded on a 7.5% (v/v) polyacrylamide gel, electrophoresed, and then blotted onto a nitro-cellulose membrane. Figure 4.7 shows the Silver-stained protein gel (Panel A) and the corresponding Western blot (Panel B). As expected, the CsCl-purified GroEL-containing T7 phage (lane 7) showed two distinct bands (60-kDa and 100-kDa) on the Western blot, whereas the same hybrid bacteriophage precipitated with 1% SDS (lane 6), showed a single 100-kDa band, corresponding to the 10B-GroEL fusion protein. The AhpC-containing T7 bacteriophage, precipitated with 1% SDS in the absence or presence of PMSF (lanes 4 and 9), showed no 60-kDa bands in either case, whereas the absence of SDS correlates with the appearance of very faint GroEL bands. This finding indicates that GroEL co-purifies with the T7 phage independently of the nature of the displayed fusion protein, but can be removed by precipitation of phage with 1% SDS prior to the CsCl density gradient centrifugation.

4.3 DISCUSSION

In the 1970s, a number of laboratories reported the isolation of *E. coli* mutants that blocked growth of either bacteriophage λ or T4, specifically at the level of head assembly, and bacteriophage T5 at the level of tail assembly (Georgopoulos *et* al., 1973; Sternberg, 1973; Zweig and Cummings, 1973). The *groE* mutants were so named because they blocked bacteriophage λ growth (gro), and the first bacteriophage λ mutants able to propagate on these mutant bacteria were shown to have amber mutations in gene E, whose product is the major capsid protein of bacteriophage λ, hence the designation groE (Georgopoulos *et* al., 1973; Sternberg, 1973). The previous genetic experiments of Fayet and collaborators (1989) demonstrated that both the *groES* and *groEL* genes are absolutely essential for *E. coli* viability under all growth conditions tested. This type of approach has been very recently extended to show that the *groES* gene can be deleted in the presence of the *31* gene of bacteriophage T4 or the *cocO* gene of bacteriophage RB49 (Ang *et* al.,

2000). Not only can the bacteriophage-encoded genes completely substitute for *groES* in *E. coli* growth, but also can replace *groES* for both bacteriophage λ and T5 growth as well. These genetic studies were substantiated by *in vitro* experiments that demonstrate that these bacteriophage-encoded polypeptides can replace GroES in the folding of all tested protein substrates of GroEL (Ang *et* al., 2000).

In this chapter, we explored whether CsCl-purified T7 bacteriophage, containing the *S. typhimurium* GroEL protein fused in-frame with the 10B minor capsid protein of the phage, and expressed onto the phage surface, contained co-purifying cellular GroEL. GroEL from the *E. coli* BLT5615 T7 host appeared on Western blots of CsCl-banded recombinant T7 phage as a 60-kDa band along with the anticipated 100-kDa band, which corresponds to the fusion protein. The S.tag-T7 phage purified through a CsCl step gradient gave also a 60-kDa band on the Western blot (see Figure 4.1).

Previous studies have shown that patterns of T7 proteins synthesis can be established using SDS-PAGE and pulse labelling with [^{35}S]-methionine (Studier and Maizel, 1969). Using this approach, a protein appears on an autoradiogram only if it is being synthesised during the period of the pulse, but disappearance of a band does not necessarily relate to the physical loss of that protein and can be explained by abolition of further synthesis. In the present study, we explored whether a 100-kDa fusion protein was synthesised, using this strategy. The 10B-GroEL fusion protein from the recombinant T7 phage was expected to be visualised after 10 minutes of incubation of the hybrid phage with the host cells, as a late T7 protein. However, the autoradiogram (see Figure 4.2) showed the lack of autoradiographic signal for the 100-kDa band corresponding to the fusion protein, and there was no difference to the autoradiogram for the T7-S.tag control phage. Thus, the inability to detect the presence of the 100-kDa band corresponding to the 10B-GroEL fusion protein on the autoradiogram of the radio-labelled late proteins from the hybrid T7 bacteriophage may be explained by the small proportion (less than 10% of the total capsid protein) represented by the fusion protein. Other factors may also have had an influence. For example, the infection of irradiated *E. coli* BLT5615 host by the

hybrid T7 phage, under the conditions used, may be anomalous. It has been reported that, using this approach, DNA synthesis is delayed and greatly reduced, with the maximum rate being less than 15% of normal and the total incorporation less than 10%. Moreover, the onset of lysis is delayed approximately 5 minutes and the period of lysis is prolonged thereby diluting the signal. In addition, the final burst size is known to be very low using this approach and probably produces less than one infective particle per cell (Studier and Maizel, 1969).

Removal of GroEL from the purified T7 phage was initially examined by a second CsCl-centrifugation, but this method proved unsuccessful (see Figure 4.3). Addition of serine-protease inhibitors throughout the T7 purification protocol improved the general outcome by protecting the expressed 10B-GroEL fusion protein from action of cellular proteases, yet the presence of the 60-kDa band on Western blots is still visible. This also supports the view that the 60-kDa band on Western blots of T7 purified bacteriophage, is not the result of 10B-GroEL degradation by cellular proteases, but the result of co-purification of bacterial GroEL with the T7 phage. However, this would not exclude the action of non-serine protease activity.

As a further attempt to remove GroEL from the purified T7 phage, the addition of ATP and Mg^{2+} during phage precipitation was explored. It is known that interactions between polypeptides and GroEL are nucleotide-dependent: ATP binding and hydrolysis by GroEL control the binding of polypeptide/GroES and the folding/release of the polypeptide (Rye *et* al., 1997). In the present study, precipitation of the hybrid T7 phage with ATP did not completely remove the GroEL chaperonin, possibly indicating a strong interaction between GroEL and the phage. The addition of GroES in addition to ATP and Mg^{2+} upon phage precipitation with 10% PEG, may well have been sufficient, as GroES can induce the release of GroEL from the phage or the surface-exposed fusion protein (Thain *et* al., 1996), but this possibility was not explored. Very recently, Rohman and Harrison-Lavoie (2000) developed an ingenious method for the complete removal of the contaminating GroEL based on addition of urea-denatured proteins to compete with the expressed protein for binding to GroEL. However, the protein denaturants urea

and guanidine greatly affect phage viability and so, cannot be used in the present situation. This study did find that addition of 1% SDS upon phage precipitation with 10% PEG successfully removed the co-purifying bacterial GroEL, possibly through disruption by the anionic detergent SDS, of the protein complex between the phage and bacterial GroEL. This provides an efficient method for removing associated GroEL from CsCl-banded T7 bacteriophage.

The hybrid T7 bacteriophage which expressed onto its capsid a fragment of the AhpC protein from *S. typhimurium,* fused in-frame with the capsid protein 10B of the phage, was used in this study as a control system for the T7-GroEL bacteriophage. A Western blot of the T7-AhpC phage (see Figure 4.7) purified in the presence of 1% SDS, either in the presence or absence of a protease inhibitor (PMSF), showed the lack of the 60-kDa band, and a faint 60-kDa signal for the phage purified in the absence of PMSF. These findings may suggest that cellular GroEL co-purifies with the T7 phage regardless of the protein expressed on the capsid, can be removed by precipitation with 1% SDS, and addition of a protease inhibitor throughout the purification protocol prevents contamination of phage with this chaperonin.

Because the GroEL-containing T7 phage was used over the course of this investigation in immunisation studies on mice, it was important to avoid any contamination of the T7 bacteriophage with bacterial proteins. In case of co-purification of GroEL protein from *E.coli* with the recombinant T7 phage, which exposes on its surface *S. typhimurium* GroEL fused to the 10B phage protein, immunisation with this product could induce immune responses in mice, directed not only to the *S. typhimurium* GroEL, but also to *E. coli* GroEL. In this case, the levels of anti-GroEL antibodies in the immunised mice could be monitored incorrectly.

The evidence provided by this study supports the fact that cellular GroEL co-purifies with the T7 bacteriophage and also that, this bacterial chaperonin attaches to the T7 phage capsid. The primary function of GroEL is to ensure that polypeptides fold

properly in the cell and this chaperone acts primarily by binding to reactive surfaces of polypeptides, which are hydrophobic (Fenton *et* al., 1994), thus preventing aggregation and promoting proper folding. Thus, attachment of cellular GroEL to the hybrid T7 phage, regardless of which polypeptide is expressed on its capsid, may relate to the primary function of GroEL as a chaperonin. However, the formation of stable interactions between the expressed GroEL-10B fusion protein and the molecular chaperone GroEL may result in the co-purification of the cellular protein with the T7 phage. This cannot be discerned from the present data, however. Moreover, binding of cellular GroEL to the GroEL-containing T7 hybrid bacteriophage may well be tighter than to the other hybrid bacteriophages, since GroEL naturally forms a barrel-shaped multimer. Early genetic studies identified the *E. coli groES* and *groEL* genes whose mutations blocked the growth of bacteriophage λ, T4, T5 and 186 (Zeilstra-Ryalls *et* al., 1991), their encoded polypeptides being involved in the phage assembly process. Moreover, the host co-expression of GroEL and GroES chaperonins was shown to increase the titre of the packaged M13 bacteriophage (Soderlind *et* al., 1995). Additionally, based on the very recent findings (Ang *et* al., 2000) that some phage capsid proteins may substitute for GroES in folding of protein substrates of GroEL, the attachment of cellular GroEL to the T7 phage is further emphasised. However, unlike several other phages, T7 was shown to be able to propagate in *groE* mutants (Molineux, 1999), but such mutants may not have been completely devoid of GroEL function.

CHAPTER 5

Study of the T7 bacteriophage as a vector to deliver bacterial proteins to the immune system of the host

5.1 INTRODUCTION

Infection by both typhoidal and non-typhoidal serovars of *S. enterica* remains a considerable global health problem (see Chapter 1). The emergence of multiple antibiotic-resistance among *S. typhi* strains and hence, the increased incidence of typhoidal mortality (Gupta, 1994), have increased the need of developing effective vaccine strategies to minimise or eliminate *S. enterica*-related diseases. In addition, *S. enterica* infection in animals is a common occurrence and has economic importance in agriculture and animal husbandry (Coynault *et* al., 1996). A vaccine against *S. enterica* infection in animals, may also limit the spread of *S. enterica* infection to humans via the food chain (Maurice, 1994).

Early studies on vaccines against *S. enterica* employed whole cell preparations (reviewed in Plotkin and Orenstein, 1999). However, apart from the inconvenience of using a multiple dose regimen, which may be difficult to implement in developing countries (Bloom, 1989), these vaccines induced minimal production of secretory IgA antibodies (Forrest *et* al., 1991; Nisini *et* al., 1993), a weak cell-mediated immune response (D'Amelio *et* al., 1988), and short-lived protection (Collins, 1974; Chatfield *et* al., 1992). The low efficacy of these vaccines prompted the search for improved immunisation procedures. Attenuated live salmonellae have played a major role in recent vaccine development, as they generate effective long-term protection due to their ability to elicit both humoral and cell-mediated immunity. The greater degree of immunity induced by live attenuated organisms has been attributed to their ability to persist in the host (Chatfield *et* al., 1992; Dougan, 1994; Ivanoff *et* al., 1994; Mastroeni *et* al., 1996). However, field trials demonstrated that live attenuated vaccines may produce symptoms of typhoid fever in vaccinees (Miller *et* al., 1993; Ivanoff *et* al., 1994) and may need multiple doses to sustain protective immunity (Ivanoff *et* al., 1994; Plotkin and Bouveretlecam, 1995).

Attenuated *S. typhimurium* cells produce stronger cell-mediated immunity and greater immunological protection in mice than killed cells (Collins, 1974; Mastroeni *et* al., 1993). This protection was attributed to the production and recognition of bacterial proteins induced in response to the host environment (Kagaya *et* al., 1992). Preliminary work on other bacterial species, such as *M. tuberculosis*, has investigated the use of mycobacterial proteins in the development of a subunit vaccine (Andersen and Heron, 1993; Andersen, 1994; Gelber *et* al., 1994; Horwitz *et* al., 1995; Silva *et* al., 1996; Tascon *et* al., 1996). It was shown that proteins which are selectively secreted by replicating bacteria, and which are also believed to be released when the bacteria reside within the macrophages, generate strong cell-mediated responses and are thought to be an important factor in the elimination of *Mycobacteria* from the host. Vaccination of mice with *M. tuberculosis* culture filtrates was found to prime T cells against a broad spectrum of these proteins (Andersen and Heron, 1993; Andersen, 1994; Horwitz *et* al., 1995), and produce protection at a level equivalent to that produced by the BCG vaccine (Andersen and Heron, 1993).

Other studies have employed the intracellular pathogen *L. pneumophila*, which produces a fatal form of pneumonia. It has been demonstrated that guinea pigs injected with the Major Secretory Protein (MSP), a heat shock protein homologue of GroEL, showed 80-85% survival three weeks after virulent bacterial challenge, and the preparation was more effective than dead cells (Blander *et* al., 1990; Blander and Horwitz, 1991, 1993). However, MSP itself is not directly involved in virulence, thus, protection does not always correlate to the immune recognition of virulence determinants. Such studies showed that any polypeptide that has the potential to initiate an immune response in the presence of a pathogen may be effective in inducing protective immunity, and can be seen as a candidate for a subunit vaccine (Blander and Horwitz, 1991). The most important property of these polypeptides is that, when suitably administered, they are capable of inducing a T_H1 type of immune response (Galdiero *et* al., 1990, 1993, 1995, 1997; Kagaya *et* al., 1992; Gupta *et* al., 1996a).

Very few *S. enterica*-derived polypeptides capable of inducing protection against a virulent bacterial challenge have been identified so far. When BALB/c mice were injected intra-peritoneally with KatG, a stress-induced catalase, 50% protection against a virulent strain of *S. typhimurium* was achieved after three weeks. In a separate study, *S. typhimurium* outer membrane proteins (OMPs) have been subcutaneously injected into BALB/c mice and proved to induce protective immunity against a lethal bacterial challenge (Udhayakumar and Muthukkaruppan, 1987). However, while proteins associated with pili and flagella, and the KatE protein from *S. typhimurium*, have been shown to induce a DTH reaction, they have not been found to be protective (Kagaya *et* al., 1992; Gupta *et* al., 1996a).

For practical use, subunit vaccines are usually preferred over live vaccines, as live vaccines present a greater risk of vaccine-induced complications. However, live vaccines are easier to produce and can elicit long-lasting immunity after only a single immunisation. In addition to being less reactogenic than live vaccines, recombinant subunit vaccines have a tendency to be less immunogenic than their conventional counterparts, particularly when highly purified. The contaminants found in some subunit vaccines may aid the inflammatory process, and thus boost immune response. This problem might be overcome by employing one of the many new types of adjuvants that are becoming available (Dertzbaugh, 1998), to improve immunogenicity and regulate the balance between T_H1 (mostly cellular) and T_H2 (mostly humoral) immune responses (Gupta and Siber, 1995; Vogel, 1995; Gupta *et* al., 1996).

Many recent studies have attempted to use novel adjuvants, such as native or recombinant cytokines, in combination with subunit vaccines to induce T_H1 immunity (see Chapter 1). IL-12 is normally produced by macrophages and B cells upon stimulation by microorganisms or their products, and is believed to influence the formation of a T_H1 type of response (Hendrzak and Brunda, 1995; Trinchieri, 1995; Abbas *et* al., 1997) and promote macrophage activation through IFN-γ (Hendrzak and Brunda, 1995; Trinchieri, 1995). Native or recombinant IL-12 has been used as an adjuvant with soluble protein antigens, and proved efficient in

sustaining memory and effector T_H1 cells *in vivo* (Bliss *et* al., 1996; Mountford *et* al., 1996; Noll and Autenrieth, 1996; Jankovic *et* al., 1997; Arulandam *et* al., 1999; Park *et* al., 2000; Stobie *et* al., 2000). Treatment of mice with recombinant IL-12 has been shown to increase resistance against salmonellosis (Kincy-Cain *et* al., 1996).

5.1.1 GroEL as major component of a *Salmonella enterica* vaccine

Heat shock proteins are widely distributed and highly immunogenic molecules. Hsp60 has been shown to be an immunodominant antigen of *Mycobacteria* and other microorganisms (Kaufmann, 1991; Jones *et* al., 1993a). Mycobacterial Hsp60 has been used as a carrier molecule for conjugated vaccines (Barrios *et* al., 1992; Perraut *et* al., 1993) and as an adjuvant (Peterman et al., 1993). The ATP-treated molecule can be covalently linked or loaded with peptides, which will elicit specific immunity without the need for another adjuvant (Barrios *et* al., 1992; Perraut *et* al., 1993; Ciupitu *et* al., 1998). This effect may be explained by Hsp60-induced stimulation of β-chemokine production from dendritic cells, macrophages, and $CD4^+$ and $CD8^+$ T cells (Lehner *et* al., 2000).

The importance of GroEL in generation of immune responses during infection by *S. typhimurium* has been demonstrated initially by Buchmeier and Heffron (1990a), and later, antibodies specific to *S. typhi* GroEL have been found in sera from patients with typhoid fever (Tang *et* al., 1997). Even though the predominant antibody response to *S. enterica* is directed towards the surface of bacteria, specific immune responses to GroEL as well as other intracellular proteins have been recorded (Brown and Hormaeche, 1989; Buchmeier and Heffron, 1990a; Tang *et* al., 1997). Taylor (1997) assessed the immunogenicity of *S. typhimurium* GroEL and AhpC during infection in mice. Both GroEL and AhpC induced specific cell-mediated and humoral immunity. When mice were challenged subcutaneously with purified GroEL or AhpC at days 33 and 104 post-infection, a significant increase in footpad swelling was observed, suggesting the presence of memory T cells (Taylor, 1997). In contrast to the results with AhpC, the antibody response to GroEL, although it developed less rapidly, was of a greater magnitude and duration, with

substantial antibody levels present at days 28 to 114 post-infection. This suggested that the kinetics of a humoral response to these two proteins could be different. The immunogenicity of GroEL has been confirmed by the appearance of antibodies after injection of the protein into uninfected mice (C. J. Inchley, personal communication).

Genetically modified bacteriophage offer an alternative method of immunisation with proteins such as GroEL. They are highly immunogenic, and specific anti-phage antibodies are rapidly induced (Inchley, 1969; Inchley and Howard, 1969; Greenwood *et* al., 1991; Meola *et* al., 1995; Manoutcharian *et* al., 1999; Grabowska *et* al., 2000; Zuercher *et* al., 2000). As previously shown, the key advantage of this type of immunogen is that no adjuvant is required for its application (Manoutcharian *et* al., 1999). In the present study, the hybrid T7 bacteriophage expressing *S. typhimurium* GroEL protein fused to the 10B capsid protein of the phage (Chapter 3), has been employed as a vehicle to deliver GroEL to the immune system of BALB/c mice, to investigate the ability of this construct to induce anti-GroEL immunity.

5.1.2 AhpC as a candidate in *Salmonella*-vaccine design

Oxidative stress is known to widely damage the bacterial cell (reviewed in Farr and Kogoma, 1991), and the ability of *S. enterica* to withstand the oxidative killing mechanisms of macrophages was demonstrated by the derepression of multigenic responses (Demple, 1991). For example, hydrogen peroxide stress induces at least 30 proteins, of which 9 are under OxyR regulatory control. The OxyR regulon includes alkyl hydroperoxide reductase (Ahp), as well as catalase (KatG), glutathione reductase (GorA), and a non-specific DNA-binding protein (Dps) involved in protection of DNA against oxidative damage (Christman *et* al., 1985).

The biochemical characterisation of *S. typhimurium* alkyl hydroperoxide reductase (Ahp) showed that this enzyme consists of two subunits, a small (26-kDa) subunit, AhpC, with peroxidase activity, and a larger (56-kDa) subunit, the flavoprotein

AhpF (Jacobson *et* al., 1989). In living cells, elimination of alkyl hydroperoxides is particularly important since they can initiate lipid peroxidation chain reaction and consequently propagate free radicals, leading to DNA and membrane damage (Halliwell and Gutteridge, 1984). In *S. typhimurium*, loss of the specific regulator OxyR, or the genes it regulates, makes the cells extremely susceptible to peroxide stress (Christman *et* al., 1985; Almiron *et* al., 1992; Buchmeier *et* al., 1995). Using the Mud*lux* reporter system, it has been shown that the *ahpC* gene is transcribed during the interaction of *S. typhimurium* with macrophages (Francis and Gallagher, 1993; Francis et al., 1997). Similarly, it has been reported that *ahpC* is expressed by *Mycobacterium* species during their interaction with macrophages (Dhandayuthapani *et* al., 1994). Very recently, it has been demonstrated that AhpC is a major antigen constitutively and highly expressed in *M. avium*, without the bacteria being submitted to oxidative stress. Infection of goats with *M. avium* induced production of antibodies against AhpC and strong IFN-γ responses (Olsen *et* al., 2000). Previous studies have shown that the *S. typhimurium* AhpC protein is antigenic in rabbits (Storz *et* al., 1989). More recently, it has been shown that while the AhpC polypeptide is not essential for the virulence of *S. typhimurium* in BALB/c mice, it is recognised as an antigen during infection (Taylor *et* al., 1998), resulting in positive antibody responses at 14 and 28 days post-infection. Purified AhpC was also found to elicit a significant inflammatory reaction (DTH) in infected mice at 33 days and 104 days post-infection, a time at which the bacteria have been cleared from the host (Mastroeni *et* al., 1993). AhpC is expressed by *S. typhimurium* during infection of BALB/c mice and constitutes a target for the immune system.

In order to further investigate its immunogenicity, this study has employed the hybrid T7 bacteriophage expressing a large fragment of *S. typhimurium* AhpC protein on its capsid, as a delivery vehicle of this protein to the immune system of BALB/c mice. By virtue of the presence of *E. coli* GroEL from the phage host attached to the hybrid T7 bacteriophage through the purification protocol, regardless of the protein expressed (Chapter 4), the AhpC-containing recombinant T7 phage has been used as a control in immunisation studies on mice, compared to the GroEL-containing T7 bacteriophage. Levels of antibodies mainly against GroEL, but also

against AhpC, have been monitored after a two-dose regimen for intravenous immunisation of BALB/c mice with purified hybrid T7 bacteriophages.

Induction of a significant antibody response directed towards the protein expressed on the T7 phage capsid (*i.e., S. typhimurium* GroEL or AhpC) would give a clear indication, in terms of future vaccine design, about the use of the T7 bacteriophage as an efficient vector able to deliver bacterial immunogenic proteins to the immune system of the host. Moreover, it would provide information about the immunogenicity of the T7 bacteriophage compared to other immunogens previously used, such as the purified proteins administered in the presence of alum as an adjuvant, and attenuated bacterial strains (*i.e., S. typhimurium aroA*$^-$ 1344 [MPG479]; Taylor, 1997).

5.2 RESULTS

5.2.1 Elaboration of an ELISA test

The ELISA protocol initially described by Cavanagh and McBride, (1997), was optimised in the present study in order to determine serum antibody levels to GroEL and AhpC induced after immunisation of mice with hybrid T7 bacteriophages (see Chapters 3 and 4). All washes and other general procedures were carried out as described in Chapter 2. Initially, in a preliminary test (Assay 1), a 96-well plate was coated with purified *S. typhimurium* GroEL for 4 hours at 37^0C, at a concentration of 1 μg/ml in coating buffer (Lauritzen *et* al., 1994). Blocking was performed by addition of blocking buffer (1% [w/v] skimmed milk in washing buffer) at 37^0C for 1 hour. Subsequently, the plate was incubated overnight at 4^0C with doubling dilutions of mouse serum samples, assayed in duplicate. Dilutions of rabbit polyclonal anti-GroEL antibodies (Sigma, UK) were included as a positive control. HRP-conjugated anti-immunoglobulin antibody with specificity for either mouse or rabbit immunoglobulins (SAPU, Scotland, UK), was added at a dilution of 1:500 in

blocking buffer. After incubation for 30 minutes at 37^0C, the wells were washed, and then incubated for 15 minutes at 37^0C with 0.1 mg/ml *O*-phenylenediamine and 0.012% H_2O_2 in development buffer (24.5 mM citric acid monohydrate and 52 mM Na_2HPO_4, pH 5.0). The reaction was stopped with 2M H_2SO_4 and absorbance was measured at 492 nm.

Negative controls (blocking buffer instead of both antibodies) and second step controls (no first antibody) were routinely included. Also included as standards to confirm the effectiveness of the assay, were pooled normal BALB/c serum and pooled day 28 serum from mice immunised with the attenuated *S. typhimurium* strain MPG 479 (Taylor, 1997). Positive wells were identified as those having an OD greater than the mean of the second step control plus three standard deviations. Assuming a normal distribution, the probability of a negative result falling above this value is less than 0.01%. This method was preferred to the regression analysis of Crowther (2001). Results are given in Table 5.1.

Subsequently, an optimised ELISA (Table 5.1) was developed from preliminary protocol, to increase its sensitivity. Previously, Taylor (1997) concluded that uninfected mice may have been exposed to GroEL or a related antigen prior to infection. Thus, a dilution of 1:100 of pooled normal mouse sera gives a positive reaction (un-published data) in a Western blot against GroEL, while at a dilution of 1:200 a positive reaction is also occasionally seen (Taylor, 1997). Based on these findings, it was expected that the titre of anti-GroEL antibodies for normal mouse sera would be in the region of 100 to 200. Thus, the anti-GroEL antibody titre of normal sera obtained in the preliminary test at a value below 8 (2^3) suggests that the protocol was not sufficiently sensitive. As a consequence, conditions employed in the preliminary ELISA test were modified in order to provide the test with a higher sensitivity (Chapter 2). Briefly, after coating the plate overnight at 4^0C with 1 μg/ml purified *S. typhimurium* GroEL, and washing as before, blocking was performed for 5 hours at room temperature (22^0C). Mouse sera were applied and incubated overnight at 4^0C. After a further wash, HRP-coupled anti-immunoglobulin antibodies were added and incubated for 30 minutes at 37^0C. Incubation with the

Table 5.1 – Evaluation of the ELISA test elaborated in this study.

Assay	Conditions of incubations					
	Coating	**Blocking**	**Mouse antibody (serum sample)**	**HRP-coupled anti-Ig antibody**	**Substrate for HRP**	**Outcome for each sample analysed in duplicate (titre of anti-GroEL antibodies)**
1 (Preliminary)	1μg/ml; 4 hours at 37^0C	1 hour at 37^0C	Overnight at 4^0C	30 minutes at 37^0C	15 minutes at 37^0C	Positive control: > 16384, > 16384 Normal mouse serum: < 8, < 8 Anti-*aroA*$^-$ serum: 256; 256
2 (Optimised)	1 μg/ml; overnight at 4^0C	5 hours at room temperature (22^0C)	Overnight at 4^0C	30 minutes at 37^0C	15 minutes at room temperature (22^0C)	Positive control: > 16384, > 16384 Normal mouse serum: 64, 128 Anti-*aroA*$^-$ serum: 2048, 4096

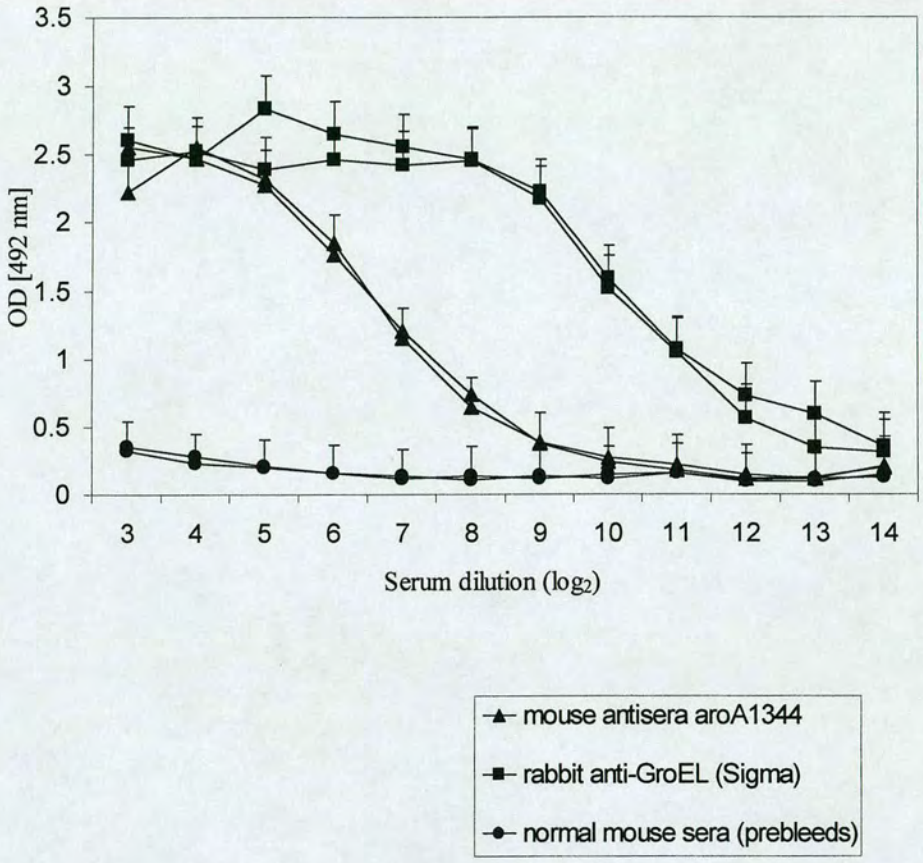


Figure 5.1

Anti-GroEL antibodies measured by an optimised ELISA.

The optimised ELISA has been performed as described in the text. The absorbance readings (O.D.) corresponding to each sample dilution (expressed as $\log_2$) are plotted. The endpoints for each mouse serum sample (tested in duplicate) can be assessed from this graph using the method described in the text. They correspond to the values given in Table 5.1. The buffer control was negative (OD < 0.01). Error bars correspond to standard deviation.

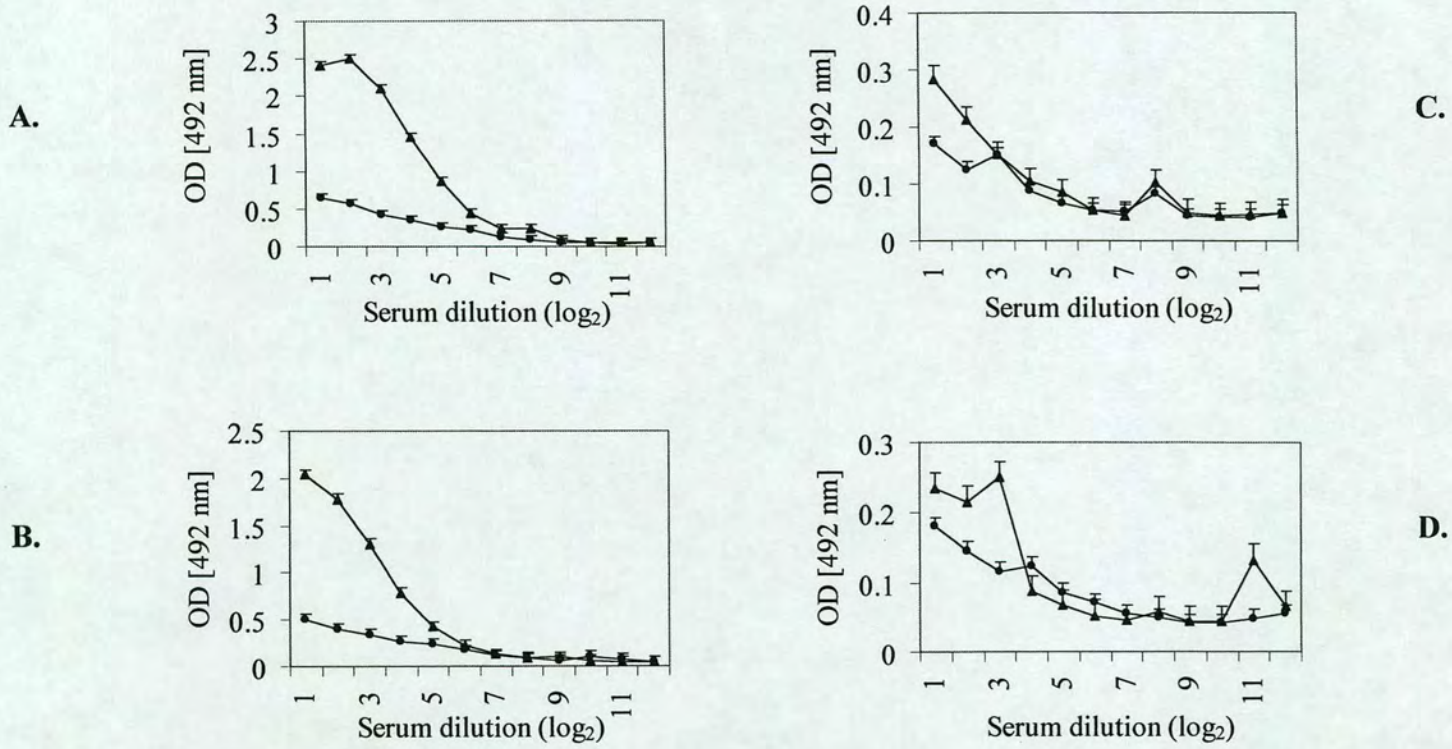


Figure 5.2 – Titration of coating antigen.

The purified *S. typhimurium* GroEL protein was titrated as described in the text before finalising the optimised ELISA procedure used in this study. The absorbance readings (O.D.) corresponding to each serum dilution (expressed as $\log_2$) are plotted. Panel A.; 1μg/ml GroEL; panel B, 0.5 μg/ml GroEL; panel C, 0.1 μg/ml GroEL; panel D, 0.05 μg/ml GroEL. Error bars represent standard deviations.

HRP substrate was for 15 minutes at room temperature, in a dark chamber. Results from this optimised test relating anti-GroEL antibodies levels in tested samples, are plotted as a $\log_2$ of the serum dilution against the measured absorbance (O.D.) (see Figure 5.1).

According to the optimised test, the titre of anti-GroEL antibodies for normal mouse serum falls between 2^6 (64) and 2^7 (128) (Table 5.1), a level which corresponds to the predicted value. Further, the titre of anti-GroEL antibodies for the mouse anti-*aroA*$^-$ serum (at day 28 post-infection) was found to fall beyond 2^{11}, a level which better supports the previous studies (Taylor, 1997).

The coating antigen must be titrated to obtain an optimal assay (Crowther, 2001). Therefore, the coating *S. typhimurium* GroEL antigen was applied in dilutions of 1 μg/ml, 0.5 μg/ml, 0.1 μg/ml, 0.05 μg/ml, and the test carried out as described for the optimised ELISA (Assay 2, Table 5.1). Results are illustrated in Figure 5.2 and confirm that the optimal coating concentration for GroEL remains 1 μg/ml, as lower concentrations do not increase the sensitivity of the test. Based on the results obtained from the optimised test (Assay 2), the procedure described for this ELISA test is used throughout the present study, mainly for determination of antibody levels induced in mice after injection with hybrid T7 bacteriophages.

5.2.2 Examination of antibody responses of mice infected with the hybrid T7 bacteriophage

The aim of this section was to determine whether the T7 bacteriophage could be used as a vector for immunisation of mice with bacterial proteins. This was addressed by using recombinant T7 bacteriophage which express on their capsid either GroEL or AhpC from *S. typhimurium* fused in-frame with the 10B minor protein of the phage (see Chapter 3 and Chapter 4 of this thesis).

After an initial pre-bleed, four groups of five female BALB/c mice, aged 4-6 weeks (15-20 g) were injected intravenously (i.v.) with 10^{10} pfu of CsCl-purified hybrid T7

phage in 100 µl of phosphate buffered saline (PBS) from the stock preserved at 4^0C. Groups A and B were injected with T7-GroEL and groups C and D with T7-AhpC (Table 5.2). The immunising dose for the hybrid T7 phage was chosen because it had been previously shown (Inchley, 1969; Inchley and Howard, 1969) that a single intravenous injection with 10^8 pfu of T4 bacteriophage induced a rapid antibody response against the phage. Because the T7Select System (Novagen Inc., U.S.A.) used in this study provides only 1-0.1 copies of the bacterial protein expressed on each capsid, the immunising dose was increased one hundred times, to 10^{10} pfu hybrid T7 per mouse. The T7 hybrid bacteriophage was administered in two separate intravenous injections 14 days apart. After infection with the hybrid T7 bacteriophage, mice were supplied with a commercial pellet diet and water *ad libitum*. Ten days after the first phage injection (day 10), and six and twelve days after the second phage injection (days 20 and 26), mice were bled and individual mouse sera were tested for antibodies by ELISA.

It has been shown in Chapter 4 of this thesis that cellular GroEL from the *E. coli* host attaches to the phage and co-purifies with it. Mice were therefore immunised both with a normal phage preparation (groups B and D) or a preparation from which bacterial GroEL had been removed (groups A and C). Removal of the contaminating GroEL from the hybrid T7 phage was performed by precipitation of phage with 10% PEG solution containing 1% SDS, and CsCl-purification in the presence of 0.5 mM PMSF (see Chapter 4). The experimental details are summarised in Table 5.2.

Background antibody levels to GroEL in normal mouse sera

Previous studies have suggested a level of background immunity to *S. typhimurium* proteins in normal laboratory mice (Brown and Hormaeche, 1989). More recently, Taylor (1997) showed that purified GroEL, injected subcutaneously (s.c) into footpads of uninfected mice, induces a transient increase in footpad thickness, suggesting previous immunological recognition of GroEL, or previous encounter with cross-reacting antigens. Taylor (1997) also showed the presence of anti-GroEL antibodies in pooled normal mouse sera (pre-immune sera) by Western blot analysis. An occasional weak positive response was reported for the presence of anti-GroEL

Mouse Group	Treatment (Schedule of T7 immunisation)		Comments
	Day 0	Day 14	
A	T7/GroEL/PMSF/SDS (*) (10^{10} pfu/mouse; i.v.)	T7/GroEL/PMSF/SDS (*) (10^{10} pfu/mouse; i.v.)	T7 from which the exogenous GroEL was removed
B	T7/GroEL/PMSF (**) (10^{10} pfu/mouse; i.v.)	T7/GroEL/PMSF (**) (10^{10} pfu/mouse; i.v.)	T7 containing the cellular GroEL attached to the phage
C	T7/AhpC/PMSF/SDS (***) (10^{10} pfu/mouse; i.v.)	T7/AhpC/PMSF/SDS (***) (10^{10} pfu/mouse; i.v.)	T7 from which the exogenous GroEL was removed
D	T7/AhpC/PMSF (****) (10^{10} pfu/mouse; i.v.)	T7/AhpC/PMSF (****) (10^{10} pfu/mouse; i.v.)	T7 containing the cellular GroEL attached to the phage

Table 5.2

A summary of the experimental protocol for examining the generation of an antibody response specific to either GroEL or AhpC, after immunisation of mice with the hybrid T7 bacteriophage.

Key: (*) T7 hybrid phage containing *S. typhimurium* GroEL, purified in the presence of 0.5 mM PMSF and precipitated with 1% SDS (T7/GroEL/PMSF/SDS); (**) T7 hybrid phage containing *S. typhimurium* GroEL, purified in the presence of 0.5 mM PMSF (T7/GroEL/PMSF); (***) T7 hybrid phage containing a fragment from *S. typhimurium* AhpC, purified in the presence of 0.5 mM PMSF and precipitated with 1% SDS (T7/AhpC/PMSF/SDS); (****) T7 hybrid phage containing a fragment from *S. typhimurium* AhpC, purified in the presence of 0.5 mM PMSF (T7/AhpC/PMSF).

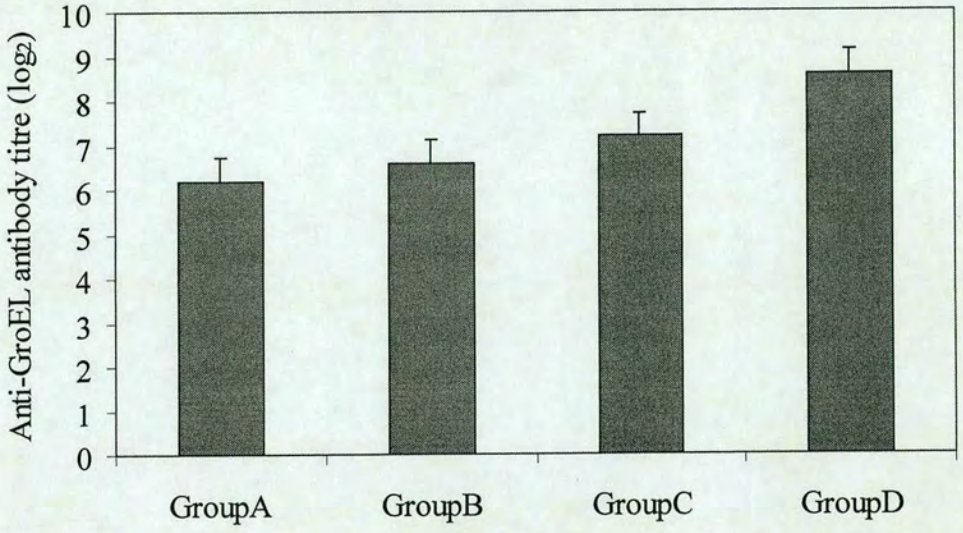


Figure 5.3

Background anti-GroEL antibody levels in all groups of mice used in the hybrid T7 immunisation study.

Mice (five per group) were bled prior to being injected with the hybrid T7 bacteriophage and the titre of anti-GroEL antibodies in pre-immune (normal) mouse individual sera was determined by an ELISA test, as previously described. Groups A, B, C, and D of mice were given different immunising hybrid T7 bacteriophages, as specified in Table 5.2. Error bars represent standard deviations.

antibodies using a dilution of 1:200 of normal mouse sera. This result has been more recently confirmed by experiments in our laboratory, which found a positive result for the presence of anti-GroEL antibodies, by performing a Western blot with a dilution of 1:100 from pooled normal mouse sera (unpublished data).

Prior to starting the experiments outlined in this chapter, all the mice (groups A, B, C, D) were bled and the sera analysed for the presence of antibody specific to GroEL by ELISA. This would determine whether the mice had previous exposure to GroEL or a cross-reacting antigen. Anti-GroEL antibody titres in normal mouse sera were found to fall near to 2^7 and 2^8, and were plotted as shown in Figure 5.3. there are no significant differences ($p<0.05$) regarding the titre of anti-GroEL antibodies in pre-immune sera, between the four groups tested in this study, as shown by analysis of variance ($p = 0.14$).

Anti-GroEL antibody responses in mice immunised with the hybrid T7 bacteriophage

Mice were given two intravenous injections 14 days apart, of the hybrid T7 bacteriophage (Table 5.2). Primary anti-GroEL antibody responses were analysed by ELISA 10 days after the first injection, and secondary responses were analysed on days 20 and 26. The ELISAs followed the optimised protocol, and the cutoff value specific to each plate, was calculated as before. Multiple tests were performed for each individual antiserum, in order to verify the reproducibility of the assay. Assuming a normal distribution of data, the paired Student's t-test was employed to analyse the different treatments. Group A mice, immunised with the *S. typhimurium* GroEL-containing hybrid T7 phage, from which the contaminating GroEL from the *E. coli* T7 host was removed, showed a very small increase in levels of specific antibody 10 days after initial immunisation ($p = 0.024$), and 6 and 12 days after challenge ($p = 0.003$, $p = 0.014$, respectively), when compared with the background (day 0) levels (see Figure 5.4, Panel A). Group B mice was immunised with the *S. typhimurium* GroEL-containing T7, contaminated with the cellular *E. coli* GroEL. By immunisation with this T7, the anti-GroEL antibody response monitored did not show a difference between the humoral response directed towards the expressed

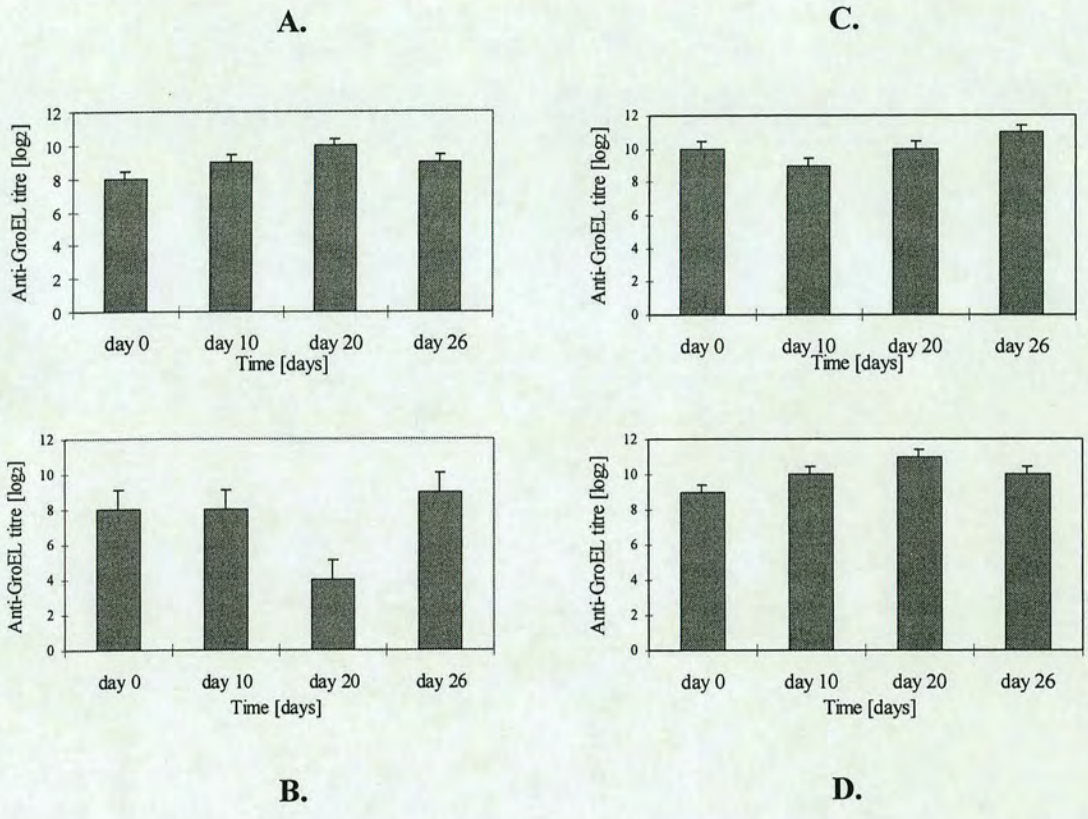


Figure 5.4

Humoral immune responses directed towards GroEL, after immunisation of different groups of mice with the hybrid T7 bacteriophage.

Key: panel **A,** shows the anti-GroEL antibody titre in group A mice after immunisation with the hybrid T7 bacteriophage T7/GroEL/PMSF/SDS (Table 5.2); panel **B,** shows the anti-GroEL antibody titre in group B mice after immunisation with the hybrid T7 bacteriophage T7/GroEL/PMSF (Table 5.2); panel **C,** shows the anti-GroEL antibody titre in group C mice after immunisation with the hybrid T7 bacteriophage T7/AhpC/PMSF/SDS (Table 5.2); panel **D,** shows the anti-GroEL antibody titre in group D mice after immunisation with the hybrid T7 bacteriophage T7/AhpC/PMSF (Table 5.2). Anti-GroEL antibody titres are represented as the mean $\log_2$ value for each group at day 0 (pre-immunisation), day 10 after the first injection, and days 20 and 26 (6 and 12 days after challenge, respectively). Error bars represent standard deviations.

protein on the phage capsid (*S. typhimurium* GroEL), and the response directed towards the cellular *E. coli* GroEL co-purified with the phage, but rather the overall anti-GroEL antibody response induced by this hybrid T7 phage. The anti-GroEL antibody response induced in the group B mice showed no increase after immunisation, as calculated by a paired t-test (see Figure 5.4, Panel B).

Group C was immunised with the *S. typhimurium* AhpC-containing T7 bacteriophage (Table 5.2), which were not contaminated with cellular GroEL from the T7 phage host, due to the SDS precipitation of the phage. Therefore, this group of mice provides a control for monitoring the anti-GroEL antibody responses in this experiment. However, the anti-GroEL antibody titres determined for the individual antisera collected from group C show a significant increase between day 0 and day 26 ($p = 0.044$; paired t-test). Figure 5.4 (Panel C) illustrates the kinetics of anti-GroEL antibodies induced in this group of mice. Group D was immunised with the *S. typhimurium* AhpC-containing hybrid T7 phage which contained traces of *E. coli* GroEL co-purified with the hybrid T7 phage (Table 5.2). However, there was no significant anti-GroEL antibody response induced in this group of mice after immunisation, as calculated by the paired t-test (see Figure 5.4, Panel D).

Examination of the background antibody levels to AhpC in normal mouse sera

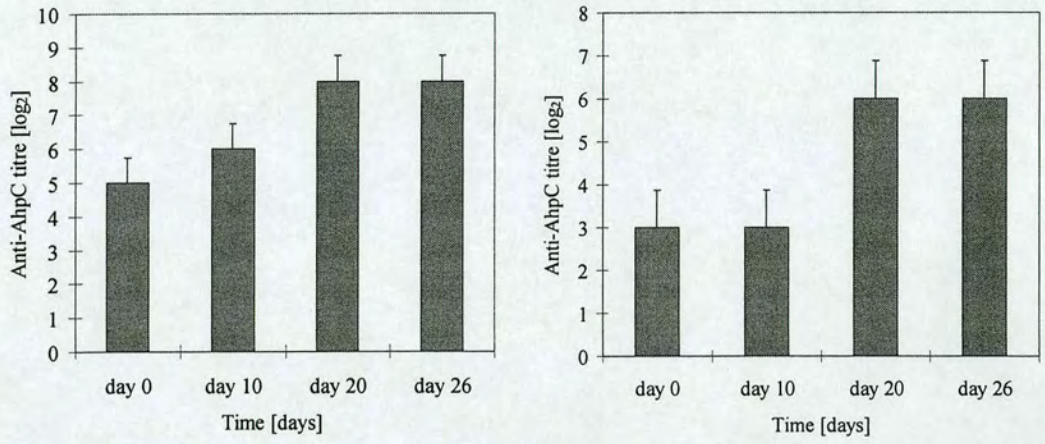
Pre-immune (day 0) sera from mice in groups C and D were assayed for background levels of anti-AhpC antibody. The ELISA test performed for measurement of anti-AhpC antibody in these mice followed the optimised protocol described for the anti-GroEL antibody ELISA, with the modification that the coating antigen used was *S. typhimurium* AhpC purified by C. Aspinwall.. Because Taylor (1997) has previously demonstrated, by performing a Western blot analysis, that a dilution of 1:200 of pooled mouse antisera collected 28 days post-infection by an attenuated *S. typhimurium* strain (MPG479), gave a positive result for detection of anti-AhpC antibodies, these sera were used as a positive control for the test.

The results show that there is a detectable amount of background anti-AhpC antibody in the normal mice sera (see Figure 5.5). Additionally to the results

presented here it must be noted that previous work performed in our laboratory (Taylor, 1997) has shown that pooled pre-immune mice sera diluted 1:200, failed to give a positive result for the detection of anti-AhpC antibodies in a Western blot analysis. Since pooled sera were analysed in this experiment, it was impossible to evaluate whether the antibody responses investigated were true for all the mice in the studied group, or if the results reflected cage- or even mouse-specific phenomena. As such, the results provided by this present study should be more accurate for the groups of mice under investigation.

Anti-AhpC antibody responses in mice immunised with the hybrid AhpC-containing T7 bacteriophage

Mice immunised with the hybrid T7 bacteriophage expressing a large fragment (18.6 kDa) from *S. typhimurium* AhpC fused in-frame with the 10B capsid protein of the phage (Chapter 4), were analysed for increases of anti-AhpC antibody in primary (day 10) and secondary (days 6 and 12 after challenge) immune responses (Figure 5.5). Each individual serum was analysed in duplicates. Figure 5.5 (Panel A) shows the anti-AhpC humoral immune responses determined for group C after injection with the hybrid T7 phage purified through a CsCl step gradient and precipitated with 1% SDS. This phage is not contaminated with the cellular GroEL protein, and therefore provides a control system for the entire experiment, regarding the immunogenicity of hybrid T7 bacteriophage, and its potential as a vector for the delivery of bacterial immunogenic proteins to the immune system of the host. By performing a paired t-test between the collected groups of data, it has been found that there is a significant statistical difference between the mean $\log_2$ anti-AhpC antibody titres on days 0 and 20 ($p = 0.0092$), and days 0 and 26 ($p = 0.0094$), showing that two injections of AhpC-T7 can induce a measurable anti-AhpC response. There is also a significant difference for the levels of anti-AhpC antibody between days 10 and 20 ($p = 0.0042$), and between days 10 and 26 (p 0.0015), suggesting that the initial injection served to prime the mice and that a secondary response was initiated after challenge. However, there is only an eight-fold increase in antibody between days 0 and 20, and a four-fold increase between days 10 and 20. There is no significant increase between days 20 and 26 ($p = 0.186$).



A. **B.**

Figure 5.5

Anti-AhpC antibody responses in mice immunised with the hybrid AhpC-containing T7 bacteriophage.

Key: panel **A,** shows the anti-AhpC antibody titre in group C mice after immunisation with the hybrid T7 bacteriophage T7/AhpC/PMSF/SDS (see Table 5.2); panel **B,** shows the anti-AhpC antibody titre in group D mice after immunisation with the hybrid T7 bacteriophage T7/AhpC/PMSF (see Table 5.2). Data are expressed as the mean $\log_2$ titre for each group. Vertical bars represent the standard deviation. Mice were bled at days 0, 10, 20 (6 days post-challenge), and 26 (12 days post-challenge).

Group D, immunised with the hybrid T7 phage expressing the fragment of *S. typhimurium* AhpC, and contaminated with the cellular *E. coli* chaperone GroEL (purified without SDS precipitation), showed an eight-fold increase in anti-AhpC antibody titre after challenge (Figure 5.5, Panel B). Overall, there were significant differences between the mean $\log_2$ titres determined on days 0 and 20 ($p < 0.001$), between days 0 and 26 ($p < 0.001$), and between days 10 and 26 ($p < 0.001$) (paired t-test). These findings confirm that two injections of T7 bacteriophage expressing *S. typhimurium* AhpC are able to induce a detectable antibody response directed towards AhpC, although the size of the response, even after challenge, is relatively small.

The humoral immune response directed towards the T7 phage particle and induced after immunisation with the hybrid T7 bacteriophage

The development of an antibody response directed towards the T7 phage particle following the immunisation schedule previously described (Table 5.2), was determined by examining the pooled sera collected from mice at day 0 (pre-immune sera), day 10 after the first injection, and days 6 and 12 after the second injection (days 20 and 26), through a Western blot analysis. This gave an indication about antibody responses specific for components of the T7 phage particle, after this was utilised as a vector for the delivery of bacterial proteins to the immune system of the host.

An SDS-polyacrylamide gel (7.5% [v/v]) (Figure 5.6, Panel A) was loaded with approximately 0.25 μg purified *S. typhimurium* GroEL (lane 1) and with approximately 0.24 μg purified *S. typhimurium* AhpC (lane 3), in order to detect any immune response directed towards these proteins after immunisation with the hybrid T7 bacteriophage. Lanes 2 and 4 were loaded with purified T7 hybrid bacteriophage, as follows: lane 2 with purified hybrid T7 phage expressing *S. typhimurium* GroEL and precipitated with 1% SDS (approximately 0.35×10^{11} pfu/well of T7/GroEL/PMSF/SDS), which therefore is not contaminated with the cellular *E. coli* GroEL, and lane 4 with purified hybrid T7 bacteriophage expressing *S. typhimurium* AhpC and precipitated with 1% SDS (approximately 0.25×10^{11} pfu/well of

T7/AhpC/PMSF/SDS), from which the cellular GroEL was removed after SDS precipitation. Molecular mass markers (SDS-6H, Sigma, UK) of 205, 116, 97, 66, 45, and 29 kDa were included on the gel as a guide to the protein size (lane M), and then the gel was subjected to electrophoresis. This gel template was used throughout the Western blot analysis.

Equivalent gels were blotted onto nitro-cellulose and the filters were incubated in the presence of 1:200 dilutions of pooled antisera from group A mice, at day 0 (pre-immune), day 10 after the first injection, or days 6 or 12 after the second injection with the hybrid T7 bacteriophage (T7/GroEL/PMSF/SDS). The presence of specifically bound antibody was then determined by incubation with rabbit anti-mouse horseradish peroxidase-conjugated antibody. The sera for each time point showed a negative response with GroEL and AhpC. However, after a negative response directed towards components of the T7 bacteriophage at day 0 (pre-immune) and day 10, a strong positive response was detected with a T7 protein of about 36 kDa at days 6 and 12 after the second injection (Figure 5.6, Panels B and C). It must be noted that the secondary immune response specific for the T7 protein is stronger at day 6 than at day 12, suggesting that this anti-T7 humoral immune response peaks around 6 days after the second boost with the phage, and begins to decline by day 12. The result for this T7 protein suggests that the T7 bacteriophage itself is immunogenic when delivered intravenously (i.v.) into BALB/c mice.

The 36-kDa protein band for which the Western blot gives a positive reaction corresponds to the 10A major capsid protein of the phage (see Figure 5.6), which accounts for more than 60% of the mass of the phage particle. Because of the frameshifting at gene 10 of the T7 bacteriophage (Condron *et* al., 1991), the 10A and 10B T7 proteins share high structural homology, therefore it would have been expected to get a humoral immune response directed not only to 10A, but also to the 10B protein of the phage, revealed by the presence of a second band in each track (Figure 5.6, panels B and C, lanes 2 and 4). However, each T7 phage particle contains 415 molecules of 10A protein, and the 10B protein represents only 10% of this. The low copy number of the 10B protein onto the T7 bacteriophage versus the

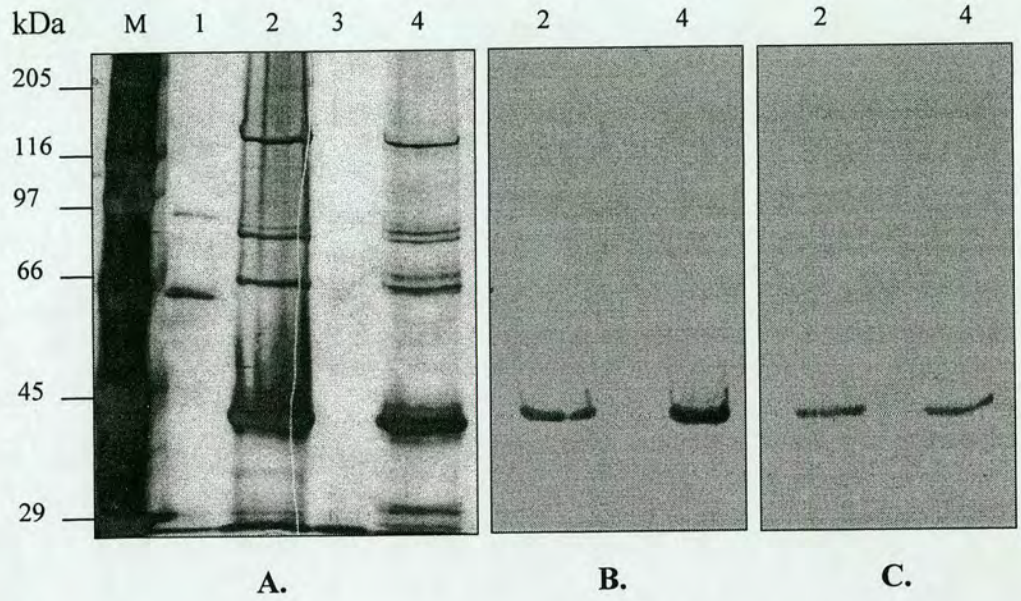


Figure 5.6 – Humoral immune responses specific to the T7 bacteriophage.

Western blot analysis of the pooled sera from group A mice (immunised with T7/GroEL/PMSF/SDS; see Table 5.2), taken on day 6 after the second injection with the T7 phage (Panel B), and day 12 after the second injection with the T7 phage (Panel C). Panel A represents a corresponding 7.5% (v/v) polyacrylamide resolving gel (7 x 8 cm) stained using the Silver stain protocol. The gel was loaded with: 0.25 μg purified *S. typhimurium* GroEL (lane 1), 0.35 x 10^{11} pfu/well of T7/GroEL/PMSF/SDS (lane 2), 0.24 μg purified *S. typhimurium* AhpC (lane 3), and 0.25 x 10^{11} pfu/well of T7/AhpC/PMSF/SDS (lane 4). Key: lane M, molecular size marker SDS-6H (Sigma). Identical gels to that described above were electrophoresed and blotted onto nitro-cellulose. Each blot was then exposed to a 1:200 dilution of the pooled sera from group A mice. The arrangement of the lanes in panels B and C is identical to that described for the Silver-stained gel.

Bleeding days	Anti-GroEL antibody titres			
	0	**14**	**28**	**49**
Assay 1	256	128	4,096	16,384
Assay 2	256	512	4,096	16,384

Table 5.3

Humoral immune responses directed toward the GroEL protein, detected in BALB/c mice antisera after immunisation with an attenuated strain of *S. typhimurium* [MPG479].

The anti-GroEL antibody titre is represented as log2 from the value determined by the ELISA test, performed for pooled mice sera at day 0 (pre-immunisation), and then at day 14, day 28 and day 49 after the i.p. injection of BALB/c mice with the attenuated *aroA*$^-$ *S. typhimurium* strain, as described by Taylor (1997). Each antiserum was tested in duplicates (Assay 1, Assay 2).

high copy number of the 10A protein, may explain why at the dilution used in the Western blot, it was impossible to get a detectable positive response with the pooled mice antisera (at days 20 and 26). Moreover, based on the high homology between 10A and 10B proteins, the recombinant proteins expressed by each T7 bacteriophage, the *S. typhimurium* GroEL fused in-frame with the 10B protein (approximately 100 kDa in total), and the fragment of *S. typhimurium* AhpC protein fused in-frame with the 10B protein (approximately 63.6 kDa in total), would have been expected to give a positive result in the Western blot analysis. Thus, these two recombinant proteins, which both share the 10B moiety, would share homology with the 10A protein and therefore, a positive band at approximately 100 kDa would have been expected to appear in lane 2 for both days 20 and 26, and also a positive band of approximately 63.6 kDa would have been expected to appear in lane 4 for both days 20 and 26 of mice antisera (Figure 5.6). However, these hybrid T7 bacteriophages constructed in this study (see Chapter 4 for details) express only 0.1-1.0 copies per virion of the bacterial protein fused with the 10B protein, a level of expression which may not be very efficient in triggering a detectable humoral immune response.

Anti-GroEL antibody responses in mice immunised with attenuated *S. typhimurium*

Previous work performed in our laboratory (Taylor, 1997) has analysed by Western blotting the immune responses directed towards the immunogenic proteins GroEL and AhpC from *S. typhimurium*, after immunisation of BALB/c mice with an attenuated strain of *S. typhimurium* (MPG479). However, ELISA tests for the detection and titration of anti-GroEL antibodies in mice antisera were not performed. As a comparison for mice immunised with T7-GroEL, BALB/c mice, immunised intra-peritoneally (i.p.) with 1.78×10^5 c.f.u. MPG479 (Taylor, 1997), were bled at intervals after infection. Pooled serum samples were assayed in duplicate for antibodies to GroEL using the optimised ELISA. The results are shown in Table 5.3, from which it can be seen that antibody titres to GroEL increased 64-fold by day 49. Although the anti-GroEL antibody levels are higher in the pre-immune (day 0) pooled mice sera than in individual sera tested in the T7 experiment, the response was greater and maintained at higher levels for a long period of time,

even at day 49 after immunisation, despite the two immunising doses used with the T7 phage.

5.3 DISCUSSION

In summary, the results presented in this chapter point out that the T7 bacteriophage can be used as an immunogen *per se*, inducing a detectable humoral immune response specific to the 10A major protein of the phage, after two intravenous doses. This response peaks six days after the second boost with the phage, and begins to decline twelve days after this. The low copy number per virion of the bacterial proteins fused with 10B, seems to make the T7 bacteriophage have a low efficiency as a delivery vector for the immunogenic bacterial proteins to the immune system of the host, being able to induce a low but detectable humoral immune response directed towards these bacterial proteins. This might contribute to subsequent protection against a pathogenic agent, such as *S. enterica*.

Protective efficacy was reported for an experimental vaccine based on secreted proteins of *M. tuberculosis* (Andersen, 1994), results that confirmed the hypothesis that a subunit vaccine based on a selection of bacterial products may provide a feasible alternative to a live recombinant carrier. Because microbial heat shock proteins are dominant antigens for the host immune responses, they may be promising candidates for the design of subunit vaccines. Noll and Autenrieth (1996) have previously shown that parenteral administration of *Yersinia* Hsp60 protein induced high anti-Hsp60-specific serum antibody responses as well as T-cell responses. Experiments subsequently performed in our laboratory (unpublished work) analysed the level of anti-GroEL antibodies induced in mice immunised with purified *S. typhimurium* GroEL protein. These experiments are outlined here. Groups of six BALB/c female mice, aged 6 weeks, were given two i.p. injections of GroEL in alum, with/without the addition of recombinant IL-12, on day 0 and day 14 (Table 5.4). Mice were bled on days 0 (pre-immune), 6 and 26, and their sera

were pooled in subgroups of 3 mice (A1, A2, B1, B2, C1, C2, D1, D2). Anti-GroEL antibodies in each serum pool were measured by the optimised ELISA, and the results are presented in Figure 5.7.

As can be observed from Figure 5.7, group A mice immunised i.p. with the purified GroEL protein in alum developed high levels of anti-GroEL antibodies after the initial injection (day 6). Titres on day 26, 12 days post challenge, were not further increased. The primary anti-GroEL antibody titres determined at day 6 after the first injection represented a 65,000-fold increase over pre-immune antibody levels. This response to protein in alum was therefore very much greater than the responses induced by immunisation with hybrid T7 bacteriophage. An explanation for these findings may be that the T7 bacteriophage used in the present study, has a very low copy number (0.1-1 copies per virion) of the immunogenic *S. typhimurium* GroEL displayed onto the phage capsid, and therefore there is a poor antigen presentation on the T7 particle. From this point of view, the T7 bacteriophage is a poor immunogen, although detectable anti-T7 antibodies are present as a secondary immune response directed toward the phage particle. The induction of high levels of antibodies specific to GroEL after immunisation with the purified protein compared to the barely detectable increase in anti-GroEL antibody titres following immunisation of mice with the hybrid T7 bacteriophage may be explained by the co-administration of alum as an adjuvant. However, T7 appears capable, at this copy level, of only inducing weak responses against inserted peptides.

By comparing antibody levels to GroEL measured after immunisation with the purified protein, to those induced after the inoculation of mice with the attenuated *S. typhimurium* strain MPG479 (Table 5.3), it can be concluded that immunisation of mice with purified GroEL, in the presence of alum, induces a higher and more rapid response. However, the level of anti-GroEL antibodies in mice infected with the attenuated *Salmonella* strain tends to persist longer, a possible consequence of the persistence of the intracellular pathogen.

Mouse Group	Treatment (Schedule of *S. typhimurium* GroEL immunisation)	
	Day 0	**Day 14**
A	50 μg GroEL per mouse precipitated 1:1 with alum, and administrated in two i.p. injections.	50 μg GroEL per mouse precipitated 1:1 with alum, and administrated in two i.p. injections.
B	50 μg GroEL per mouse precipitated 1:1 with alum, and administrated in two i.p. injections; 0.25 μg recombinant IL-12 per mouse administered i.p. in one injection.	50 μg GroEL per mouse precipitated 1:1 with alum, and administrated in two i.p. injections; 0.25 μg recombinant IL-12 per mouse administered i.p. in one injection.
C	0.25 μg recombinant IL-12 per mouse administered i.p. in one injection.	0.25 μg recombinant IL-12 per mouse administered i.p. in one injection.
D	100 μl PBS per mouse administered i.p.	100 μl PBS per mouse administered i.p.

Table 5.4

A summary of the experimental protocol for examining the generation of an antibody response specific to GroEL, after immunisation of mice with purified *S. typhimurium* GroEL protein.

Each group of mice consisted of six female BALB/c mice, 6 weeks of age. Antisera collected from sub-groups of three mice were pooled and analysed by performing ELISA tests for the presence of anti-GroEL antibodies.

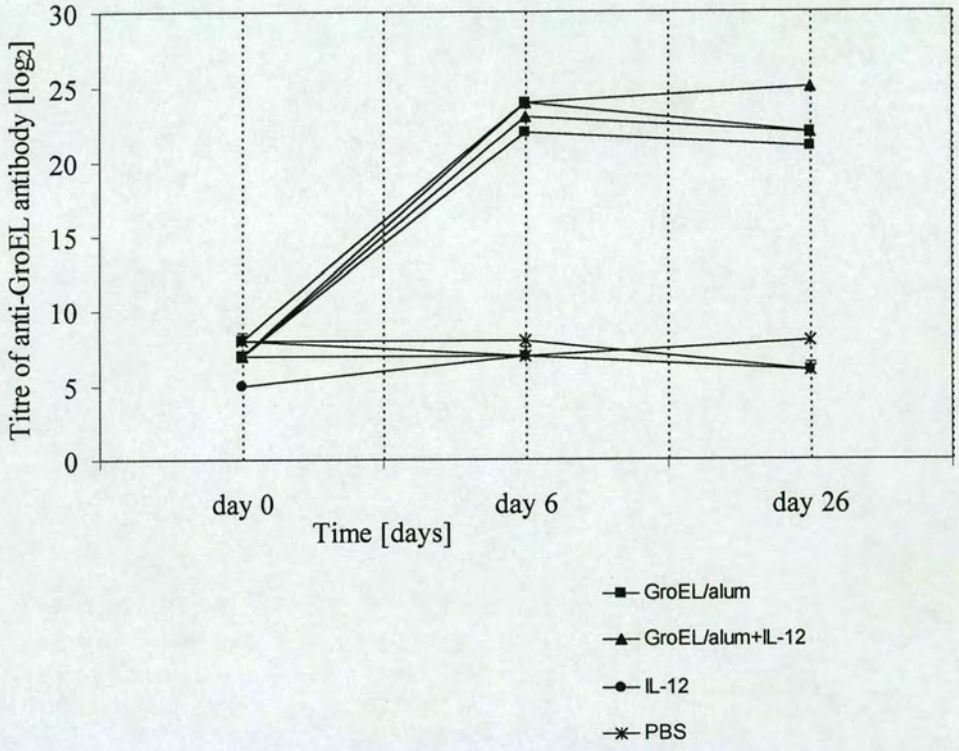


Figure 5.7

Humoral immune responses directed toward the GroEL protein, detected in BALB/c mice antisera after immunisation with the purified GroEL protein from *S. typhimurium.*

The anti-GroEL antibody titre is represented as $\log_2$ from the value determined by the ELISA test, performed for pooled mice sera at day 0 (pre-immunisation), and then at day 6 after the first injection and day 12 after the second injection (see Table 5.4 for immunisation schedule). Each group of mice (A, B, C, D) was split in two subgroups containing three mice each, and pooled sera were collected from each subgroup (A1, A2, B1, B2, C1, C2, D1, D2).

IL-12 is a potent stimulator of T_H1 responses and various studies have previously investigated the potential of native or recombinant IL-12 as an adjuvant in the administration of soluble protein antigens (Bliss *et* al., 1996; Mountford *et* al., 1996; Jankovic *et* al., 1997; Arulandam *et* al., 1999; Park *et* al., 2000; Stobie *et* al., 2000). However, different authors used different doses/routes of IL-12. For example, Noll and Autenrieth, (1996) focused on the use of IL-12 as an adjuvant in the parenteral administration of purified *Y. enterocolitica* Hsp60 protein. When IL-12 was included, significant Hsp60-specific T-cell responses were induced, together with protection against subsequent challenge with yersiniae. Therefore, the potential of IL-12 as an adjuvant in the i.p. administration of the purified *S. typhimurium* GroEL protein was also investigated (Table 5.4, Figure 5.7), as a preliminary experiment. However, no significant difference in the anti-GroEL antibody titres at day 6 after the first injection and day 12 after the second injection was found between groups A (no IL-12) and B (with IL-12). Therefore, in mice immunised i.p. with the purified *S. typhimurium* GroEL protein, with alum as an adjuvant, the recombinant IL-12 did not induce any increase in the primary or secondary humoral responses to GroEL. However, the cellular immune responses in these mice were not studied. It may be that IL-12 induced additional cell-mediated immunity due to its effects on T_H1 cells. It is well known that both humoral and cellular immune responses must be taken into account for the design of an efficient vaccine.

The icosahedral T7 phage displaying on its capsid peptides or proteins fused to the carboxyl-terminus of phage coat proteins has been used in recent studies as a model peptide delivery system. Sokoloff and collaborators (2000) reported that displayed peptides were recognised by natural antibodies and induced complement activation. Moreover, initial studies on the fate of phagocytosed T4 (Inchley, 1969) indicated that this is rapidly inactivated and broken down during first few hours after injection, but part of the constituent antigens are then localised in cytoplasmic organelles and protected from catabolism. After intravenous injection, the T4 antigen is phagocytosed by splenic macrophages, its immunogenicity persists longer than, and is not proportional to the rate of phage destruction.

The immunisation of mice with a hybrid T7 bacteriophage as a vector for the delivery of immunogenic proteins to the host immune system, such as GroEL from *S. typhimurium*, has been analysed in this study. Compared to other methods of immunisation described above, which also investigated the production of anti-GroEL antibodies, the T7 system proves to be ineffective. However, two aspects of the T7 system are promising. Firstly, the anti-AhpC responses induced by T7-AhpC, even at low copy number, were stronger than those against GroEL, though not strikingly so. Secondly, modified T7 kits are available which will allow for expression of high copy number of inserted peptide genes. These should be tested, not only for their ability to induce antibody, but also for their ability to stimulate cell-mediated immunity. An efficient vaccine to intracellular pathogens involves the participation of both humoral and cellular immunity and therefore, the next step in analysing the efficiency of a T7-based vaccine against *Salmonella* infection, would be to investigate whether the cellular immunity against *S. typhimurium* GroEL is triggered by the hybrid T7 phage, when expressed at a higher copy number on the phage capsid. By challenging the mice footpads with purified *S. typhimurium* GroEL protein and then at 24 and 48 hours measuring the footpad enlargement from these mice initially immunised with the hybrid T7 phage, would determine whether cellular immunity is induced. In case of positive results for the participation of both humoral and cellular arms of the immune responses directed toward *S. typhimurium* GroEL, then the level of protection against a subsequent *Salmonella* infection of mice would be interesting to examine, by performing an LD_{50} test for mice immunised with the hybrid T7 bacteriophage.

CHAPTER 6

Final discussion

6.1 CONCLUDING DISCUSSION

S. enterica infection contributes to the massive incidence of diarrhoeal disease recorded in the world each year (Pang *et* al., 1995, 1998). Although antibiotics have proved useful in the treatment of *S. enterica*-related illness, the emergence of antibiotic resistance makes antibiotic therapy practically useless. Therefore, a major objective is to devise suitable prophylactic therapies, such as vaccines, to reduce the incidence of a *S. enterica* infection. Of relevance to this thesis is the antigenicity and immunogenicity of the GroEL protein from *S. typhimurium*, which can therefore be used as a base-component in the construction of a *Salmonella* vaccine studied in a mouse model.

Much of the recent work into developing an effective vaccine to *S. enterica* has focused on the use of attenuated bacterial strains (Chatfield *et* al., 1992; Ivanoff *et* al., 1994; Ivanoff and Levine, 1997). Live attenuated bacteria provide much greater protective immunity than killed cells, but this ability has been attributed to the fact that the live bacteria express immunogenic proteins in response to the host environment (Kagaya *et* al., 1992). A polypeptide-based vaccine is more likely to have wide-scale use in humans and animals and be safer, less reactogenic, and may induce stronger protective immunity than whole-cell vaccines. The immunological relevance of *S. typhimurium* GroEL was initially demonstrated by Buchmeier and Heffron (1990a) and later on, high titres of antibodies specific to *S. typhi* GroEL were detected by Tang and collaborators (1997) in sera from patients with typhoid fever. It is now well known that the successful elimination of *S. enterica* from its host involves the generation of a T_H1 response (Kagaya *et* al., 1992; Mastroeni *et* al., 1993), and therefore, any form of a polypeptide-based vaccine would have to stimulate the T_H1-type of immune response to be effective. Moreover, recent work performed in our laboratory by Taylor (1997) has shown that the *S. typhimurium*-derived GroEL protein is able to induce significant humoral and DTH responses, suggesting that this protein is recognised in the context of a T_H1 response.

Bacteria, such as *S. typhimurium*, are capable of persisting within the host, and therefore there is greater probability for the antigens derived from bacteria to be displayed over a long period. Moreover, factors associated with the bacterium are likely to stimulate the immune system and therefore, bacteria have natural adjuvant properties. Thus, bacterial components such as LPS are known to up-regulate immune responses by stimulating B cells to undergo isotype switching, such that they produce complement fixing antibodies, or by activating macrophages (Baldridge and Wood, 1997). Much of the work into the ability of polypeptides to generate protective immunity showed that protection is most effective in the presence of an adjuvant (Kagaya *et* al., 1992; Andersen, 1994; Gelber *et* al., 1994; Noll and Autenrieth, 1996). For example, the T_H1 response-associated cytokines, such as IL-12, were successfully used as adjuvants to skew the immune responses to the desired phenotype (Noll and Autenrieth, 1996). However, data from a preliminary experiment reported in this study, reveal no significant difference in levels of anti-GroEL antibody titres between sera from BALB/c mice immunised i.p. with the purified *S. typhimurium* GroEL protein, and sera from mice administered the same protein and recombinant IL-12, concomitantly. Analysis of antibody isotypes might have characterised the responses more precisely and shown changes which were not revealed by the basic ELISA. As opposed to the studies involving immunogenicity of proteins administered in association with various adjuvants, this study focuses mostly on the ability of a protein, such as *S. typhimurium* GroEL, when expressed onto a phage vector, such as the T7 bacteriophage, to induce a humoral response in BALB/c mice.

During this study, BALB/c mice were immunised intravenously with the hybrid T7 bacteriophage, which expresses on its capsid the GroEL protein from *S. typhimurium* fused in-frame with the 10B capsid protein of the phage, and humoral immune responses directed toward this GroEL protein were monitored. Due probably to the low copy number estimated at less than 10% of the total capsid protein, this immunising agent stimulated barely detectable levels of antibody against GroEL. These responses were significantly weaker than the anti-GroEL antibody levels

induced in mice injected i.p., either with the purified GroEL protein from *S. typhimurium* in the presence of alum, or with the *aroA*⁻ attenuated *S. typhimurium* strain (MPG479). Levels of antibody in mice immunised with purified GroEL were approximately 4 orders of magnitude greater than the maximum detected in mice immunised with T7-GroEL, probably because of insufficient antigen presentation by the hybrid T7 bacteriophage, and also because the purified protein was administered in the presence of alum as an adjuvant. Although the anti-GroEL antibody response determined in mice immunised with the purified protein precipitated with alum showed higher levels than the anti-GroEL antibody response determined in mice immunised with the *aroA*⁻ attenuated *S. typhimurium* strain, the latter was more persistent.

The findings indicate that the hybrid T7 bacteriophage in the form used here is not an efficient immunising agent compared to the purified GroEL protein administered with alum as adjuvant, or to the attenuated *S. typhimurium* strains. Nevertheless, detectable anti-10A secondary antibodies (measured at day 20 and day 26) directed towards the major capsid protein of the phage, were present after immunisation with the hybrid T7 bacteriophage, suggesting that the T7 bacteriophage is immunogenic by itself, and it is possible that expression of a high copy number of the immunogenic protein would be more efficient in the induction of a high and persistent humoral immune response. The immunogenicity of T7-AhpC is encouraging in this respect. Because the efficiency of a vaccine is measured by its capacity to induce both humoral and cellular immune responses, it would be relevant to investigate also the induction of cellular immune responses directed toward the *S. typhimurium* GroEL protein expressed onto the phage capsid, once a high copy number transfected phage has been established.

After construction and sequencing of the hybrid T7 bacteriophage, this was purified prior to immunising BALB/c mice. This work is the first to report the contamination of the CsCl-purified hybrid T7 phage particle, with the exogenous, cellular GroEL protein from the *E. coli* BLT5615 host of the phage. Due to its chaperone function, the GroEL protein assists in correct folding of proteins assembled within the host

cell, and also is involved in early phases of bacteriophage morphogenesis (Zeilstra-Ryalls *et* al., 1991). As a result, the cellular GroEL co-purifies with the hybrid T7 bacteriophage through the CsCl step gradient purification protocol, regardless of the heterologous protein expressed on the phage capsid, and may therefore alter the measurements of anti-GroEL antibody levels induced by immunisation of mice with this phage. However, in order to eliminate the cellular GroEL protein attached non-specifically to the phage, this study provides a solution for the de-contamination of the T7 CsCl-purified particle, by precipitation of the phage with 1% SDS, an anionic detergent which has the capacity of disrupting protein complexes, and leaves the phage viable.

After previous studies performed in our laboratory (Taylor, 1997), identified a single homologue of *groEL* on the *S. typhimurium* chromosome, and localised the *groEL* gene at 93-96 minutes on *S. typhimurium* chromosome, the present work is the first to report the full DNA sequence of the *groEL* gene from *S. typhimurium* SL1344 chromosome. The nucleotide sequence shows 98% identity with *S. typhi groEL* gene, and 93% identity with the *E. coli groEL* gene, whereas the putative protein sequence corresponding to the sequenced *groEL* fragment shows 50% amino acid identity with the human molecule, and 100% identity with the *S. typhi* GroEL protein. These findings support further localisation of immunodominant regions on the *S. typhimurium groEL* gene, by construction of nested deletions at both carboxy- and amino-terminal ends of the fragment fused in-frame with the GST protein from *S. japonicum*, purification of these truncated fusion proteins, and performing ELISA tests using sera from mice immunised with the purified *S. typhimurium* GroEL protein.

GroEL remains a promising candidate for consideration in any potential vaccine against *S. enterica*-related illness. Previous work has shown that GroEL homologues from various bacterial species, such as *M. tuberculosis* (Silva *et* al., 1996), *M. leprae* (Gelber *et* al, 1994), *Y. enterocolitica* (Noll and Autenrieth, 1996), *L. pneumophila* (Blander and Horwitz, 1993), and *H. pylori* (Ferrero *et* al., 1995) are highly immunogenic and induce some degree of protection against lethal challenge with the

respective bacteria. Moreover, the work performed in our laboratory and referred to above has provided evidence that GroEL from *S. typhimurium* is highly immunogenic and induces both long-lived T and B cell responses, the efficiency of which depends on the method of immunisation. The Hsp65 mycobacterial protein (GroEL type) has been used efficiently in previous studies (Barrios *et* al., 1994) as an adjuvant by exerting a strong *in vivo* helper effect when conjugated to synthetic peptides or bacterial oligosaccharides and given in the absence of any adjuvants. More recently, Lehner and co-workers (2000) have demonstrated that Hsp60 covalently linked to antigens are potent agents enhancing serum antibodies and $CD4^+$ T cell proliferative responses when administered by the systemic route, as well as secretory IgA and IgG antibodies when given by the mucosal route in primates.

Nevertheless, the use of a GroEL-based vaccine is controversial (Kaufmann, 1990). The relationships between bacterial heat shock proteins and autoimmunity were first disclosed in the *Mycobacteria*-induced model of adjuvant arthritis: passive transfer of a T cell clone responding to mycobacterial Hsp60 evoked disease in naïve recipient animals (reviewed by Shoenfeld and Isenberg, 1988). Subsequent studies also concluded that high levels of IgG anti-human Hsp60 autoantibodies found in autoimmune diseases, such as reactive arthritis (RA), systemic lupus erythematosus (SLE), Reiter's syndrome, arise primarily as a consequence of the humoral immune responses to *E. coli* GroEL through the recognition of cross-reactive epitopes (Handley *et* al., 1996). The suggested explanation is that exposure to normal environmental bacteria expressing GroEL, or very similar Hsp60, but not mycobacterial Hsp65, may be responsible for the induction of most human Hsp60 autoantibodies. However, anti-human Hsp60 autoantibodies are not indicative of autoimmune disease in RA and SLE, since they are found in similar titre in normal sera (Handley *et* al., 1996).

Also, it was demonstrated that a subset of patients with a history of *Salmonella* infection developed reactive arthritis. The persistence of bacterial antigens in arthritic tissue and the isolation of *Salmonella* or *Yersinia* reactive $CD8^+$ T cells

from the joints of patients with RA support the etiological link between Gram-negative bacterial infection and autoimmune disease. Models proposed to account for the link between infection and autoimmunity include inflammation-induced presentation of cryptic self-epitopes, antigen persistence and molecular mimicry. Recent studies (Lo *et* al., 2000) identified an immunodominant epitope derived from *S. typhimurium* GroEL, which is presented by the class Ib molecule Qa-1 and is recognised by $CD8^+$ cytotoxic T lymphocytes induced after natural infection. This suggests the involvement of MHC class Ib molecules in infection-induced autoimmune recognition and indicate a mechanism for the etiological link between Gram-negative infection and autoimmunity.

GroEL is remarkably immunogenic, despite its high degree of evolutionary conservation. Cross-reactivity between bacterial GroEL and the homologous mammalian host protein was initially demonstrated on the mycobacterial GroEL (Kaufmann, 1990). Alignments of the *M. tuberculosis* and human GroEL homologues suggest 48% identity between these two proteins (Jindal *et* al., 1989). When the protein sequence of GroEL was divided into stretches of 25 amino acids, and these fragments were put into a database of protein sequence, 86 human polypeptides were identified, which exhibited high sequence similarity to many of these fragments. Furthermore, 19 of these polypeptides were involved in autoimmune diseases such as insulin-dependent diabetes and rheumatoid arthritis (Jones *et* al., 1993a), although a causative relationship has yet to be shown. Moreover, the use of a *S. enterica* GroEL homologue as a basis for designing a vaccine against *Salmonella* would probably give rise to concerns about its safety, as the *S. typhi* GroEL shares 75% identity with the *M. tuberculosis* homologue, and also shares 51% identity with the human derivative (see Figure 1.2 for an alignment).

The mechanism of Hsp-induced autoimmunity may be explained by the participation of different factors *in vivo*. First, infectious agents containing homologous Hsp60 proteins could induce an anti-self immune response through molecular mimicry in susceptible individuals (Schoel and Kaufmann, 1996). Additionally, vaccination

(particularly with heat-inactivated bacteria) may contribute to the development of anti-Hsp antibodies. Second, viral infection may result in incorporation of Hsp60, or at least of Hsp60-derived peptides, into the envelope of the budding virus (Bartz *et* al., 1994). Arising from viral infections, Hsp60 could also become immunogenic as a result of structural alteration or post-translational modification (Schattner and Rager-Zisman, 1990). Third, exposure to endogenous Hsps or mimicry proteins might induce immune reactions against Hsp60, or endogenous Hsps may interact with other immunogenic proteins (Coulie and van Snick, 1985). For example, the natural microbial flora may influence such immune responses to various degrees, *e.g.*, GroEL of *E. coli* from the intestine.

Proteins of the Hsp60 family were initially considered to be located intracellularly in mitochondria only, where they facilitate protein translocation and act as chaperones protecting proteins from harmful enzymatic attacks during folding. Accumulating evidence now points to an additional surface localisation of Hsp60 proteins on not only eukaryotic cells (Soltys and Gupta, 1996), and in response to infection (Belles *et* al., 1999), but also on prokaryotic cells, such as *H. pylori* (Yamaguchi *et* al., 1996) and *S. typhimurium* (Ensgraber and Loos, 1992). Immune responses against Hsps could therefore provide a general basic level of protection and limit the spread of infections by acting as an initial defence mechanism that immediately interacts with microbial invaders. In contrast to this benefit, the risk of autoimmunity must be taken into consideration.

While anti-Hsp60 antibodies and γδT cells reactive with Hsp60 proteins are involved in autoimmune diseases (reviewed in Winfield and Jarjour, 1991), there is no evidence that immunisation with purified Hsp60 proteins may induce chronic autoimmune disease. In fact, to the contrary, there is evidence that immunisation with GroEL-like proteins protects against autoimmune disease. Pre-treatment of rats with a mycobacterial Hsp65 protects against adjuvant-induced arthritis and against streptococcal cell wall-induced T cell dependent polyarthritis (Bahr *et* al., 1990; Cobelens *et* al., 2000). Immunisation of non-obese diabetic mice with the Hsp65 of *M. tuberculosis* protects against spontaneous autoimmune insulin-dependent

diabetes mellitus (Funda *et* al., 1998). In other words, exposure of the immune system to bacterial antigens, such as GroEL, may well stimulate the immune system to resume control over unwanted self-reactive clones. Laboratories-OM (Geneva) have been producing *E. coli* bacterial lysates, which are used amongst others for the treatment of reactive arthritis. They are administered orally and have shown in multiple trials in RA patients a good effectiveness (Van der Zee *et* al., 1998), and recently it has been revealed that the *E. coli* Hsp60 molecule (GroEL) is one of the more prominent immunogens present in this material (Vischer and van Eden, 1994). This rather general protective potency of bacterial Hsp against arthritis seems to result from the capacity of strongly conserved sequences in the protein to activate T cells that cross-recognise the mammalian homologous Hsp sequences presented on cells at the site of inflammation (Van der Zee *et* al., 1998).

As a final conclusion, this study provides useful information about the T7 particle as an immunising agent and vector for the delivery of immunogenic protein to the immune system of the host, the full sequence of *S. typhimurium groEL* gene, and about problems encountered throughout the purification of the T7 phage particle. It is important to note also that further assessment on the cellular immune responses induced toward the protein expressed on the phage particle, would give more information about the capacity of the T7 phage to function as an efficient immunising agent in mice. Moreover, the identification of the immunoglobulin isotypes involved in the humoral immune responses, and the determination of the level of protection induced by immunisation with the high copy T7 hybrid phage, by performing an LD_{50} test, would provide a complete image about the T7 phage particle and its induced immunity in mice.

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